REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier. Claims 57, 58, 59, 60 and 64 are amended to delete portions of the claims that are directed to non-elected subject matter. Claims 54 and 61-63 are canceled without prejudice or disclaimer as directed to non-elected subject matter. Claim 57 is not canceled as PTO-326 indicates that this claim is withdrawn from consideration but the Office Action on page 2 does not include claims 57 as withdrawn. Applicants have not canceled claim 57 at this time as there appears to be confusion regarding the status of this claim. After amending the claims as set forth above, claims 38-53, 55-60 and 64-66 are now pending in this application.

1. Rejections under 35 USC 112, second paragraph

1.1 Claims 38 to 53, 55, 56, 59, 60 and 64 to 66

The Examiner states that the terms "unstable", "in a cell" and "co-precipitates" allegedly are indefinite as it is not clear what the "metes and bounds" of these terms are.

Applicants respectfully disagree with the Examiner's interpretation of that these terms are indefinite and submit that they are clear when read in light of the specification. In assessing whether claims are invalid for indefiniteness, the Court of Appeals for the Federal Circuit (CAFC) generally follows the following principle:

> If the claims, read in light of the specification[], reasonably apprise those skilled in the art both of the utilization and scope of the invention, and if the language is as precise as the subject matter permits, the courts can demand no more.

(citation omitted) *Shatterproof Glass Corp. v. Libbey-Owens Ford Co.*, 758 F.2d 613, 624, 225 USPQ 634 (Fed. Cir. 1985

Applicants direct the Examiner to the specification where the objected terms are exhaustively defined, see for example, page 5 line 4 to page 7, line 21. The experimental conditions which can be applied to define the terms are also described in detail:

With respect for the terms "unstable" and "in a cell", the specification discloses:

> "As used in accordance with the present invention, the term "stable in a cell" denotes (poly)peptides which have a half-life in a cell of at least two hours. In other words, if the amount of label incorporated into a (poly)peptide in pulse-chase experiments is not reduced to one half within less than two hours after termination of the pulse phase, the corresponding (poly)peptide is regarded stable. (Poly)peptides the half-lives of which are less than two hours or not measurable by pulse-chase experiments at all are regarded "unstable" in accordance with the present invention. Pulse-chase experiments used, e.g., to determine the stability of a compound in a biological system are well known to the person skilled in the art (see, e.g., Darnell et al. (eds.), Molecular Cell Biology, 1986, Scientific American Books, Inc., New York; Ausubel et al. (eds.), Current Protocols in Molecular Biology, 1989, Green Publishing Associates and Wiley Interscience, New York). For instance, cells may be incubated in the presence of [^{35}S] methionine for 10 to 15 minutes followed by a chase period of several hours. During said chase period cells may be harvested at different times, proteins of interest isolated, e.g., by immunoprecipitation, and the amount of incorporated label determined, e.g., in SDS-polyacrylamide gel electrophoresis (for a preferred detailed protocol see Examples 2 and 3, infra, and Schirmbeck, R. and J. Reimann, *Eur. J. Immunol.* 24 (1994): 1478). With respect to the stability "in a cell", it will be readily evident for the person skilled in the art that this phrase comprises the stability of a (poly)peptide in any compartment of the cell including, e.g., the cytoplasm, the endoplasmatic reticulum and the compartments involved in the secretory pathway, the nucleus if present, etc." (specification page 5, lines 4 to 25) (emphasis added).

Furthermore, also the term "co-precipitates" is clearly defined in the specification:

> As used in accordance with the present invention, the phrase "second (poly)peptide which co-precipitates a chaperone" describes, in general, an interaction of said second (poly)peptide with a chaperone that leads to the precipitation of both the (poly)peptide and the chaperone upon a further (prior or simultaneous) interaction of the (poly)peptide with a precipitating agent. Precipitating agents may be of various nature. However, a prerequisite they must fulfill is that the interaction with said second (poly)peptide is specific. A preferred type of precipitating agent is an antibody such as a

> monoclonal antibody or a fragment or derivative thereof. Advantageously, the interaction of the (poly)peptide with the chaperone is characterized by a binding constant that is higher than the binding constant between the chaperone and the natural counterpart of said second (poly)peptide within the fusion protein under physiological conditions within a cell. Accordingly, a (poly)peptide which co-precipitates a chaperone interacts with a chaperone in such a way that the interaction originally established during synthesis of said (poly)peptide is maintained after completion of translation and through an (artificial) precipitation step. (specification, page 5, last paragraph, to page 4, line 13) (emphasis added).
>
> The experimental conditions under which a second (poly)peptide comprised in the fusion protein of the present invention detectably co-precipitates a chaperone are as follows: During a period of about 15 min cells are incubated with a radiolabeled amino acid such that all proteins newly synthesized in this pulse period become radioactively labeled. A subsequent chase with an excess amount of the non-labeled form of this amino acid ensures that only proteins synthesized during this 15 min pulse period are radioactive and detectable by this tag during the subsequent chase period. In accordance with the present invention a detectable co-precipitation of a chaperone still has to occur after 5 min, preferably after 10 min, more preferably after 20 min, and most preferably after 30 min until to about 2 hours, preferably to about 4 hours, and most preferably to about 6 hours after the end of the pulse period/the beginning of the chase period. (specification page 6, last line to page 7, end of first paragraph) (emphasis added).

The above portions of the specification demonstrate that the Examiner's rejection is not justified since the terms "unstable", "in a cell" and "co-precipitates" are supported by the specification and clearly defined. As the claims of an application are to be read in view of the specification, it is requested that this rejection be withdrawn.

2. Rejection under 35 USC 112, first paragraph

2.1 Claims 38 to 53, 55, 56, 59, 60 and 64 to 66

Claims 38-53, 55, 59, 60 and 64-66 are rejected as allegedly failing to comply with the written description requirement. The Examiner states that the claims contain subject-matter which was not described in the specification in such a way as to reasonably convey to one

skilled in the relevant art that inventors, at the time the application was filed, had possession of the claimed invention.

Particularly, the Examiner states that the claims are directed to a peptide that is not stable in a cell which in turn is part of a fusion protein that "co-precipitates" with a chaperon. The Examiner cites page 8, lines 7-11 of the specification which the Examiner alleges defines "unstable" as "therefore, are only difficult to express per se or which can not be expressed at all" as exemplifying that the specification does not define a standard which might allow an artisan to experimentally apprehend when a protein is stable or not thus rendering the scope of the claims unclear.

Applicants respectfully disagree with the Examiner's interpretation of the portion of the specification that has been taken out of context. The Examiner refers to page 8, lines 7-11, which discusses that polypeptides for which stability can be increased are those that are expressed with difficulty or not expressed at all. However, the terms as used within the meaning of the present invention are defined in the specification on page 5, lines 4-25 and as discussed above, i.e., "(Poly)peptides the half-lives of which are less than two hours or not measurable by pulse-chase experiments at all are regarded "unstable" in accordance with the present invention," the specification does define "unstable" so that a person skilled in the art would have been able to carry out the invention over the whole claimed range of the invention. Consequently, the inventors had possession of the claimed invention at the date of filing. In view of this argument and directing the Examiner's attention to explicit support in the specification to the term "unstable," it is requested that this rejection be withdrawn.

2.2 Claims 38 to 53, 55, 56, 59, 60 and 64 to 66

Claims 38-53, 55, 56, 59, 60 and 64-66 are rejected for allegedly failing to comply with the adequate written description requirement. The Examiner states that it is allegedly not clear how the claimed invention produces products that are different from those described in Cheng et al., 1986, which according to the Examiner discloses nucleic acids that lead to the expression of the same HBV proteins and an immunological response as well as detection and vaccines.

Applicants respectfully disagree with the Examiner's basis for this rejection, and question the unorthodox use of prior art to support his position that Cheng somehow provides the basis to suggest the present invention is not supported by an adequate written description. The Examiner questions how the examples in the present application show stabilization of proteins as used in the examples have been expressed before. We refer the Examiner to the following arguments that show that the present invention and the disclosure content of Cheng et al. can be distinguished.

Specifically, Cheng et al., 1986 describes expression and immunogenicity of the Hepatitis B virus (HBV) large surface antigen (LHB_SAg; preS1-pre2-S) using a vaccinia virus-based expression system. In this system, HBV preS1 and preS2 domains were expressed in their natural form with the small (S) HBV surface antigen. The preS1-pre2-S (LHB_SAg) protein was expressed in two forms: a non-flycosylated p39 and a glycosylated p42 protein. The authors describe co-induction of preS- and S- specific immune responses. The present inventors have confirmed these findings using CMV-driven expression plasmid DNAs encoding the natural LS protein. However, the data in the present specification showed that the isolated preS1-preS2 domain (without the S) cloned into different expression units is not expressed and, thus, did not induce immune responses. The goal of the present invention is to express the isolated HBV preS domain (without the S) and to selectively induce immune responses against it.

Applicants submit that the present specification provides support to show that the claimed polynucleotides are enabled so that a person skilled in the art may practice the present invention in a repeatable manner. More specifically, the present invention is based on the observation that mutant SV40 T-Ag species with an intact N-terminus show stable binding to the constitutively expressed, cytosolic stress protein hsp73. Attachment A provided in Figures 1 and 2 a flow chart that diagrammatically depicts the following explanation. The hsp-binding sequence was mapped to the DnaJ-homologous, 77 residue N-terminal fragment (as shown Fig. 1, #1 in Attachment A), which supports the isolation of a viral, DnaJ-homologous, hsp73-capturing domain. Further, vectors have been designed for the expression of hsp-capturing fusion proteins. For example, different polypeptide sequences were cloned in frame downstream of the hsp-binding N-terminal T-Ag fragments using a multiple cloning site (MCS). The N-terminal, T-Ag-derived sequence was either a

complete hsp-capturing, DnaJ-homologous domain (>77 N-terminal T-Ag residues), or a truncated T-Ag-derived sequence (comprising e.g. the N-terminal 60 residues of the T-Ag) without detectable hsp73 binding activity. The expression vector(s) further contains the usual upstream enhancer/promoter sequence, natural or synthetic stop codons for the termination of the polypeptide synthesis, and downstream polyadenylation signal sequence (see Fig. 1; #2, in Attachment A). Intron sequence(s) are optional. Different, commercial vector constructs, such as e.g. pCI, pcDNA3 or pBMGneo, have been successfully used for expression. For more details, see Example 2 in the present specification, which discloses vectors useful in the present invention.

Further the present specification demonstrates stable expression and hsp-binding of the fusion protein expressed by the vector. More specifically, vector DNA expressing hsp-capturing or not hsp-capturing fusion proteins was transfected into cells of different species or tissue origin (see Fig. 2; #3, in Attachment A). To test the relative efficacy of expression of hsp-bound versus non-hsp-bound fusion protein(s), an identical C-terminal, protein-encoding sequence was fused in frame to either the (hsp-binding) T77 domain, or the T60 domain (not binding hsp). The cloning vector (pBLUEscript) or eukaryotic expression vector (pCI; pCDNA-3; pBMG) used was found not to be critical for the successful expression of fusion protein. The selection and design of polypeptide sequence that are fused in frame to the N-terminal T-Ag sequences was not critical. As apparent from the attached Table I (see Attachment B), many polypeptide sequences have been successfully expressed in different, transfected cell lines. Following transfection of these vector DNAs into cells, all fusion peptides were expressed (see Fig. 2; #4 in Attachment A). This was shown by immunoprecipitating the fusion protein from the lysates of transiently transfected cells using a monoclonal antibody binding the N-terminal 10 residues of the T-Ag. These immunoprecipitation studies also revealed that polypeptides with an intact N-terminal DnaJ domain (T77) were co precipitated with an equimolar amount of cellular hsp73 while polypeptides containing an truncated DnaJ domain (the T60 sequence) did not coprecipitate hsp73 (see Fig. 2; #5 in Attachment A). Furthermore, immunoprecipitation with an anti-hsp73 monoclonal antibody coprecipitated fusion proteins carrying an N-terminal T77 domain, but not fusion proteins carrying an N-terminal T60 domain. Hence, stable association with cytosolic hsp73 strictly correlated with an intact DnaJ N-terminus of the fusion protein

(for detail see: R. Schirmbeck, N. Fissolo, P. Chaplin and J. Reimann. "Enhanced priming of multispecific, murine CD8+ T cell responses by DNA vaccines expressing stress protein-binding polytope peptides." (2003) *J.of Immunology* 171: 1240 (attachment hereto as Attachment C) and R. Schirmbeck R., M. Kwissa, N. Fissolo, S. ElKholy, P. Riedl and J. Reimann. "Priming polyvalent immunity by DNA vaccines expressing chimeric antigens with a stress protein- capturing, viral J-domain." (2002) *FASEB J.* 16: 1108, (attached hereto as Attachment D).

Binding of hsp73 to the fusion proteins was stable as revealed by pulse/chase experiments. The half-life of fusion protein/hsp73 complexes was determined as 10-48 h. This was further shown by the observation that hsp73/fusion protein complexes accumulated to high steady-state levels within the cells (>2 μg/107 cells). In contrast, the half-life of fusion proteins with a non-hsp binding T60 N terminus was <6 h and these proteins did not accumulate to steady state levels in transfected cells (Fig. 2; #6 in Attachment A). Thus, hsp73 binding stabilizes expression of fusion proteins within the cell.

These findings extended the data described in Example 5 of the present invention, where it was shown that SV40 T-Ag variants with an intact N-terminal DnaJ domain (but not those with a truncated or deleted DnaJ-domain) were efficiently expressed in transfected cells. Hsp-binding of T-Ag variants or T-Ag fusion proteins facilitates expression in cells (also see Example 7 of the present invention).

Additionally, vaccination with plasmid DNA encoding Hsp-binding T-Ag variants or T-fusion proteins elicits humoral immune responses. Fusion proteins carrying an N-terminal T77 domain, but not fusion proteins carrying an N-terminal T60 domain were efficiently expressed in transfected cells. Vaccination studies showed that enhanced expression correlated with an efficient induction of antibody responses against the fusion protein in vivo. This was demonstrated using a Hepatitis B virus core domain (C79-149) fused to the T77- or T60 fragments (described in: R. Schirmbeck R., M. Kwissa, N. Fissolo, S. ElKholy, P. Riedl and J. Reimann. "Priming polyvalent immunity by DNA vaccines expressing chimeric antigens with a stress protein- capturing, viral J-domain." (2002) *FASEB J.* 16: 1108 – Attachement D).

Thus, expression of the hsp73-capturing T77/C79-149 fusion protein enhanced the immunogenicity of the C79-149 domain for B cells. These findings extend the data described

in example 8, where we show that hsp-binding SV40 T-Ag variants (but not the non-hsp bound wild type T-Ag) induce T-Ag specific serum antibodies, which further indicates that the data described in Example 9 of the present specification (for the generation of anti preS1/S2 antibodies) can be reproduced in other antigen systems.

The present inventors have provided an inexpensive and efficient means that reliably allows the expression of (poly)peptides within cells via the Hsp73/T-Ag system and successfully presents these polypeptides to the immune system. Applicants submit that this contribution to the field entitles the inventors to claim their invention in a manner which provides adequate coverage for their contribution, and applicants maintain that the disclosure supports this general principle and provides an adequate written description. It is not mandatory, and is practically impossible, to disclose in the description, each and any potential embodiment which might be derived from a generic claim.

In further support that the present invention is adequately described, applicants also provide a review article which is authored by the inventors and which will be published in the journal, *Immunological Reviews*, (see Attachment E) which further supports that the claimed polynucleotides, vector, host cells and methods are supported over the whole claimed range of the invention. Particularly, Table III of this review shows that the antigens expressed as hsp capturing, chimeric antigens have a half life of more than 12 hours. Additionally enclosed is a list of constructs 1 – 47 provided by the inventors, showing the successful production in the "hsp-facilitated expression system." (See Attachment F), thus providing additional evidence of an adequate written description of the present specification.

Applicants submit that with the above information, the enclosed supporting documents and furthermore with the disclosure of Examples 2, 5, 7 and 9 in the specification as outlined above, provide sufficient evidence that the specification provides an adequate written description. In view of these arguments and documents, it is requested that this rejection be withdrawn.

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date December 29, 2003

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ATTACHMENT A

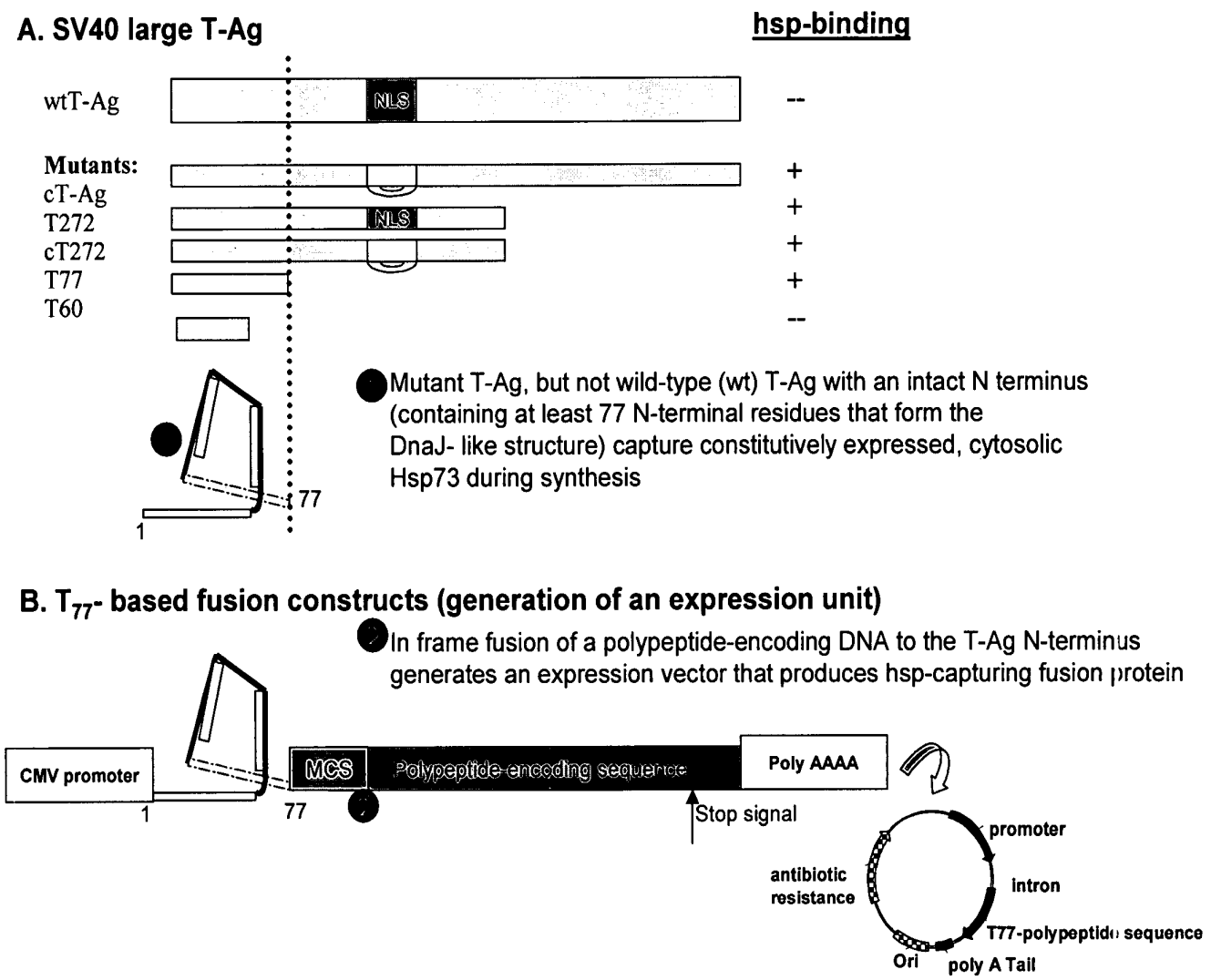
A. SV40 large T-Ag
hsp-binding
wtT-Ag
NLS
--
Mutants:
cT-Ag
+
T272
NLS
+
cT272
+
T77
+
T60
--
77
1
Mutant T-Ag, but not wild-type (wt) T-Ag with an intact N terminus (containing at least 77 N-terminal residues that form the DnaJ- like structure) capture constitutively expressed, cytosolic Hsp73 during synthesis
B. T77- based fusion constructs (generation of an expression unit)
In frame fusion of a polypeptide-encoding DNA to the T-Ag N-terminus generates an expression vector that produces hsp-capturing fusion protein
CMV promoter
MCS
Polypeptide-encoding sequence
Poly AAAA
1
77
Stop signal
promoter
antibiotic resistance
intron
T77-polypeptide sequence
Ori
poly A Tail

Figure 1

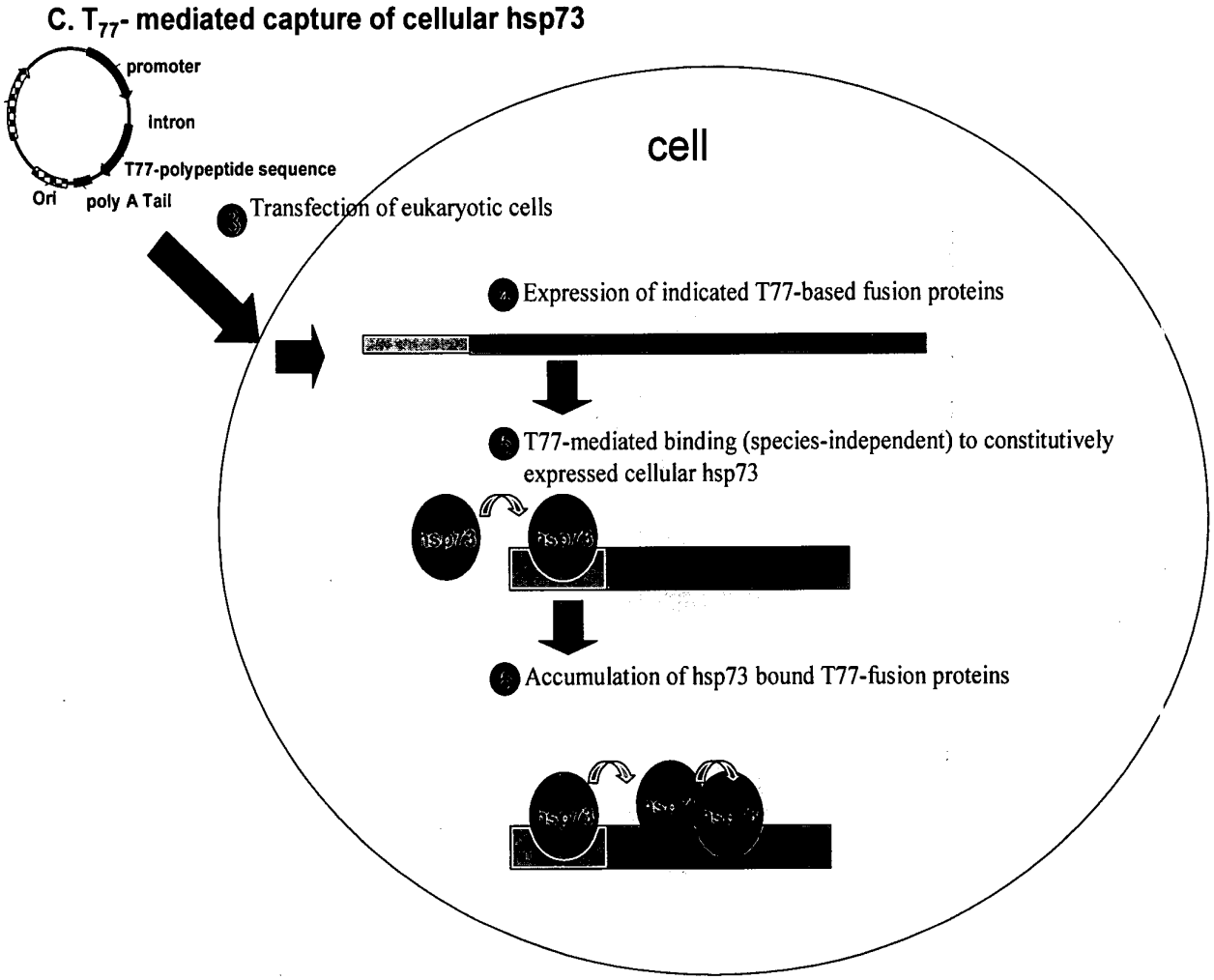
C. T77- mediated capture of cellular hsp73
promoter
intron
T77-polypeptide sequence
Ori
poly A Tail
Transfection of eukaryotic cells
cell
Expression of indicated T77-based fusion proteins
T77-mediated binding (species-independent) to constitutively expressed cellular hsp73
Accumulation of hsp73 bound T77-fusion proteins

Figure 2

ATTACHMENT B

Table I: Expression of hsp-capturing, chimeric antigens

source	antigen(s)	fragment	length
HBV	polymerase	full	832aa
HBV	polymerase	1-380	380
HBV	polymerase	351-620	270
HBV	polymerase	585-832	248
HBV	X-Ag	full	154
HBV	core	79-149	71
HBV	S	1-100	100
HBV	S	80-180	101
HBV	S	140-226	87
HBV	S	140-175	36
HBV	S	167-205	39
HBV	S	195-226	32
HBV	preS1preS2	1-163	163
HBV	S/S	S80-180 +S20-50	132
HBV	S/core	S80-180 + core 1-183	284
HBV	S/core	S80-180 +core110-183	175
HBV	S/eGFP	S140-226 +eGFP	
HDV	L-Ag	full length	214
HDV	L-Ag	1-82	82
HDV	L-Ag	73-152	80
HDV	L-Ag	141-214	75
HDV	L-Ag-eGFP	L-Ag +eGFP	444
HCV	core	Full	150
SIVmac239	RT	281-412	136
model proteins	eGFP	full length	230
	Her2 polytope	full length	>1000 106

ATTACHMENT C

Enhanced Priming of Multispecific, Murine CD8$^+$ T Cell Responses by DNA Vaccines Expressing Stress Protein-Binding Polytope Peptides[1]

Reinhold Schirmbeck,* Nicolas Fissolo,* Paul Chaplin,† and Jörg Reimann[2]*

A polytope DNA vaccine (pCI/pt10) was used that encodes within a 106-residue sequence 10 well characterized epitopes binding MHC class I molecules encoded by the K, D, or L locus (of H-2^d, H-2^b, and H-2^k haplotype mice). The pCI/pt10 DNA vaccine efficiently primed all four K^b/D^b-restricted CD8$^+$ T cell responses in H-2^b mice, but was deficient in stimulating most CD8$^+$ T cell responses in H-2^d mice. Comparing CD8$^+$ T cell responses elicited with the pCI/pt10 DNA vaccine in L^{d+} BALB/c and L^{d-} BALB/c^{dm2} (dm2) mice revealed that L^d-restricted CD8$^+$ T cell responses down-regulated copriming of CD8$^+$ T cell responses to other epitopes regardless of their restriction or epitope specificity. Although the pt10 vaccine could thus efficiently co prime multispecific CD8$^+$ T cell responses, this priming was impaired by copriming L^d-restricted CD8$^+$ T cell responses. When the pt10 sequence was fused to a 77-residue DnaJ-homologous, heat shock protein 73-binding domain (to generate a 183-residue cT$_{77}$-pt10 fusion protein), expression and immunogenicity (for CD8$^+$ T cells) of the chimeric Ag were greatly enhanced. Furthermore, priming of multispecific CD8$^+$ T cell responses was readily elicited even under conditions in which the suppressive, L^d-dependent immunodominance operated. The expression of polytope vaccines as chimeric peptides that endogenously capture stress proteins during in situ production thus facilitates copriming of CD8$^+$ T cell populations with a diverse repertoire. ***The Journal of Immunology,*** **2003, 171: 1240–1246.**

The efficient and specific stimulation of T cell responses is a priority in current vaccine research. Solutions to many unresolved problems of vaccine development against human diseases such as AIDS, tuberculosis, papilloma and herpes virus infection, chronic hepatitis B and C, and cancer, are expected to depend on the specific priming of MHC class I- and class II-restricted T cell responses against the relevant pathogen (1). Current approaches to design T cell-stimulating vaccines are based on novel adjuvants, synthetic peptides, recombinant viruses, or DNA vaccines.

With the availability of the complete sequence of the genomes of many major pathogens, the choice of Ags (and their immunogenic epitopes) has been strikingly expanded. Vaccines incorporating multiple, antigenic peptides binding to either MHC class II molecules (stimulating CD4$^+$ Th cell responses) or class I molecules (stimulating CD8$^+$ CTL responses) are an attractive choice for stimulating cellular immunity (2). This can focus CTL responses to multiple target Ags and/or multiple epitopes of a target Ag, and can provide help for priming CTL responses by codelivering class II-restricted T cell effector functions. By eliminating suppressive or immunodominant epitopes and optimizing MHC binding affinity and TCR contact, the immunogenicity of such synthetic peptide vaccines can be increased considerably.

Multiepitope vaccines can be constructed as either a synthetic peptide vaccine or as a DNA vaccine (or an expression cassette cloned into the genome of a recombinant virus). If the length of the peptide is >50–80 residues, the DNA-based approach is easier for vaccine designs, at least at the stage of experimental, preclinical vaccine designs. We therefore exploited the DNA vaccination approach to test in the mouse some aspects relevant for the design of optimal CTL-stimulating, multiepitope vaccines. Long peptides representing linear constructs of multiepitope or polytope vaccines have higher intrinsic immunogenicity than mixtures of individual peptides, although they require processing for acquiring T cell stimulatory potency (3, 4). Although combinations of epitopes can create new junctional epitopes (that are irrelevant for antipathogen immunity), their disruption by appropriate spacers between epitopes can solve this problem (4). Furthermore, the combination of a multitude of antigenic epitopes may establish novel immunodominance hierarchies that limit the immunogenicity of the subdominant epitopes. This can be overcome by codelivering an appropriate adjuvant (as shown in this report).

The N-terminal domain of the SV40 large tumor Ag (T-Ag)[3] associates with the stress protein 73-kDa heat shock protein (hsp73) through a DnaJ-like structure, i.e., the J domain (5–9). T-Ag associates with hsp73 through the conserved HPD motif and the α-helical structure of the J domain. We have reported that different mutant T-Ags show stable association with hsp73 if their N terminus is intact (10–14). The N-terminal domain of T-Ag required for hsp73 association is located within the T$_{77}$ (but not the T$_{60}$) fragment that hence contains the intact J domain (15). From this observation we derived a vector system for the efficient expression of hsp73-associated, chimeric proteins (12, 13, 15–17). In this system the T-Ag-derived J domain is fused N-terminally to different sequences from heterologous viral Ags of different origins and lengths. This allowed us to design DNA vaccines that stably express chimeric, hsp73-binding proteins at a high level. In

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[3] Abbreviations used in this paper: T-Ag, large tumor Ag of SV40; B6, C57BL/6 inbred strain; BFA, brefeldin A; c, cytoplasmic; DC, dendritic cell; FCM, flow cytometry; hsp73, 73-kDa heat shock protein; pt10, polytope vaccine used in this study.

0022-1767/03/$02.00

addition to enhancing expression, hsp70 molecules are innate adjuvants that enhance and modulate the immunogenicity of protein and peptide Ags (reviewed in Refs. 18–20). The adjuvant effect of hsp molecules seems to have many facets. Hsp induces the migration and maturation (including cytokine/chemokine release) of dendritic cells (DC) (21–27). Hsp facilitates the priming of $CD8^+$ T cell responses to peptides from tumor Ags, minor H Ags, or viruses by delivering them to processing pathways for MHC class I-restricted presentation (26, 28–39). Hsp is thus an attractive innate adjuvant in vaccine formulations to enhance its immunogenicity for CTL. We compare the immunogenicity of multiepitope DNA vaccines for $CD8^+$ T cells that express Ag associated or not associated with hsp73.

Materials and Methods

Mice

C57BL/6J (B6) mice (H-2^b), BALB/cJ mice (H-2^d L^{d+}), BALB/c^{dm2} (dm2) mice (H-2^d L^{d-}), F_1 (BALB/c × C57BL/6) mice, and F_1 (dm2 × C57BL/6) mice were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). The $H2^{dm2}$ strain has a deletion of ~140 kb that encompasses $D2^d$, $D3^d$, $D4^d$, and L^d; dm2 mice are congenic to BALB/c. Male female mice were used at 12–16 wk of age.

Cells

The H-2^d mastocytoma cell line P815 (TIB64) was obtained from American Tissue Culture Collection (Manassas, VA). The H-2^b (B6-derived) T lymphoma cell line RBL5 was obtained from Dr. H.-U. Weltzien (Freiburg, Germany). The chicken hepatoma cell line LMH was obtained from Dr. H.-J. Schlicht (Ulm, Germany)

Vector constructs

The 106-residue murine polytope sequence (Fig. 1) was obtained from a pt10-encoding Bluescript construct with primers (GAATTCATAT GTCTAGAGCCAGCAACGAGAACATG +; generating an *Eco*RI site) and (GGTACCTAAGTGCTCGGGGCCGGACAC−; generating a *Kpn*I site) and was cloned directly into a pEGFP N1 (Clontech, Palo Alto, CA; catalogue no. 6085-1)-based mammalian pCMV expression vector, generating the plasmid pCMV/pt10. In this vector Ags are expressed under human CMV promoter/enhancer control. Construction of the vectors encoding the SV40 T-Ag derived T_{60} or T_{77} fragments (*pBlue/*T_{60} and *pBlue/*T_{77}) has been described (12, 14, 15, 40). The T_{60}- and T_{77}-encoding sequences were cloned into the pCMV expression vector, generating plasmids pCMV/T_{77} and pCMV/T_{60}. The pt-10-encoding *Eco*RI-*Kpn*I fragment was cloned C-terminally (in-frame) into plasmids pCMV/T_{77} and pCMV/T_{60}, generating plasmids pCMV/T_{77}-pt10 and pCMV/T_{60}-pt10, respectively.

The polytope DNA vaccine pt10

#	sequence	epitope	from source	restriction
	M'SR			
1	ASNENMDAM	$NP_{366-374}$	Influenza nucleoprotein	D^b
2	SIINFEKL	$OVA_{257-264}$	Ovalbumin	K^b
3	TYQRTRALV	$NP_{147-155}$	Influenza nucleoprotein	K^d
4	SDYEGRLI	NP_{50-58}	Influenza nucleoprotein	K^k
5	YPHFMPTNL	$pp89_{168-176}$	Murine cytomegalovirus pp89	L^d
	TS			
6	SGPSNTPPEI	E1 $A_{234-243}$	Adenovirus 5 E1A	D^b
7	FAPGNYPAL	$NP_{324-332}$	Sendai virus nucleoprotein	K^b
8	SYIPSAEKI	$CSP_{249-257}$	P. Berghei circumsporozoite protein	K^d
9	EEGAIVGEI	$NS1_{152-160}$	Influenza nonstructural protein I	K^k
10	RPQASGVYM	$NP_{118-126}$	Lymphocytic choriomeningitis virus nucleoprotein	L^d
	PRNNLVSGPEHL[106]			

FIGURE 1. Map of the polytope (pt10) vaccine. The plasmid pCMV/pt10 encodes 10 murine, H-2^d-, H-2^b-, and H-2^k-restricted $CD8^+$ T cell epitopes. The amino acid sequences, the protein Ags and the pathogens from which they are derived, and the restricting MHC class I molecules that bind the epitopes are shown. TS, two spacer residues between epitopes 5 and 6. The C-terminal 11 residues represent an Ab-binding epitope (42). The epitopes and reference to their first published description are listed in the syfpeithi.bmi-heidelberg.com database.

Expression of polytope constructs by transfected cells

LMH cells were transiently transfected with plasmid DNA using the Ca_3PO_4 method. Briefly, 10 μg of plasmid DNA was adjusted to 0.25 M $CaCl_2$ in a volume of 500 μl and mixed with 500 μl of 2× HBS buffer (280 mM NaCl, 1.5 mM Na_2HPO_4, and 50 mM HEPES, pH 7.1). Semiconfluent cell monolayers were incubated with the DNA solution for 12 h. Cells were metabolically labeled for 12–15 h with [^{35}S]methionine 36 h after transfection, extracted with lysis buffer (120 mM NaCl, 1% aprotinin (Trasylol, Bayer, Leverkusen, Germany), leupeptin, 0.5% Nonidet P-40, and 50 mM Tris-hydrochloride (pH 8.0)) for 30 min at 4°C. Extracts were cleared by centrifugation and immunoprecipitated for T-Ag using the mAb PAB108 and protein A-Sepharose. Immune complexes bound to protein A-Sepharose were purified with wash buffer (300 mM LiCl, 1% Nonidet P-40, and 100 mM Tris-hydrochloride, pH 8.5), followed by two washes in PBS and 0.1× PBS. Immune complexes were recovered from protein A-Sepharose with elution buffer (1.5% SDS, 5% ME, and 7 mM Tris-hydrochloride, pH 6.8), processed for SDS-PAGE, and analyzed by fluorography.

DNA vaccination

For i.m. nucleic acid immunization, we injected 50 μl of PBS containing 1 μg/μl of plasmid DNA into each tibialis anterior muscle as previously described (12, 41). Intradermal injection of 2 μg of particle-coated DNA with the Helios Gene Gun system (catalogue no. 165-2431; Bio-Rad, Munich, Germany) has been described (14, 15), with the modification that immunization was performed with two shots of 300 psi.

Peptides

Synthetic peptides 1, 2, 3, 5, 6, 7, 8, and 10 listed in Fig. 1 were obtained from Jerini BioTools (Berlin, Germany). Peptides were dissolved in a DMSO stock solution at a concentration of 10 mg/ml and were diluted with culture medium before use.

Determination of splenic $CD8^+$ T cell frequencies

Spleen cells (1 × 10^7/ml) were incubated for 1 h in RPMI 1640 medium with 0.1 μg/ml of the respective peptide. Thereafter, 5 μg/ml brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO; catalogue no. 15870) was added, and the cultures were incubated for an additional 4–6 h. Cells were harvested and surface-stained with PE-conjugated anti-CD8 mAb (BD PharMingen, San Diego, CA; catalogue no. 01045B). Surface-stained cells were fixed with 2% paraformaldehyde in PBS before intracellular staining for IFN-γ. Fixed cells were resuspended in permeabilization buffer (HBSS, 0.5% BSA, 0.5% saponin, and 0.05% sodium azide), incubated with FITC-conjugated anti-IFN-γ mAb (BD PharMingen; catalogue no. 55441) for 30 min at room temperature, and washed twice in permeabilization buffer. Stained cells were resuspended in PBS/0.3% (w/v) BSA supplemented with 0.1% (w/v) sodium azide. We determined the frequencies of $CD8^+$ IFN-γ^+ T cells by FCM analyses. The mean numbers of double-positive $CD8^+$ IFN-γ^+ T cells/10^6 $CD8^+$ spleen cells from three to six individual mice are shown.

Results

Priming multispecific, murine $CD8^+$ T cell responses by polytope DNA vaccines

The plasmid pCMV/pt10 encodes 10 well-characterized epitopes binding to murine MHC class I molecules encoded by the K, D, or L locus of H-2^d, H-2^b, and H-2^k haplotype mice. Fig. 1 lists these 10 epitopes, their amino acid sequences, the protein Ag and the pathogens from which they are derived, and the restricting MHC class I molecules that bind the epitope. The pt10 DNA vaccine has been shown to prime multispecific, murine CTL responses (42).

We injected 100 μg of pCMV/pt10 DNA i.m. into H-2^b (C57BL/6, B6; Fig. 2*A*) or H-2^d (BALB/c) mice (Fig. 2*B*). No

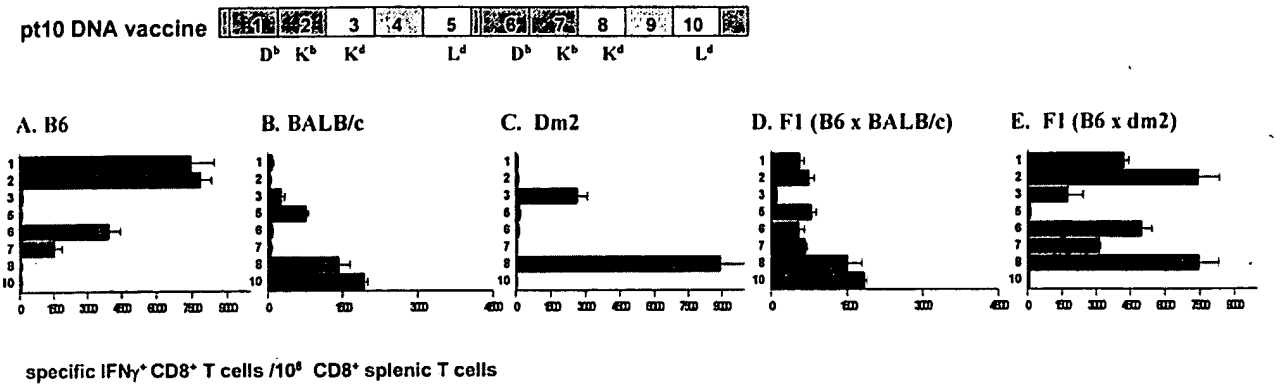

FIGURE 2. Priming pt10-specific $CD8^+$ T cell responses. B6 (*A*), BALB/c (*B*), L^{d-} BALB/c dm2 (*C*), F_1 (B6 × BALB/c) (*D*), and F_1 (B6 × dm2) (*E*) mice were vaccinated i.m. with 100 μg of pCMV/pt10 plasmid DNA. Spleen cells obtained 12 days postvaccination were restimulated for 5 h with H-2^b-restricted epitopes 1, 2, 6, and 7 and with H-2^d-restricted epitopes 3, 5, 8, and 10. T cells were surface-stained for CD8 and intracellularly stained for IFN-γ. We determined the frequencies of $CD8^+$ IFN-γ^+ $CD8^+$ T cells per 10^6 $CD8^+$ spleen cells by FCM analyses. The mean numbers of IFN-γ^+ $CD8^+$ T cells per 10^6 $CD8^+$ spleen cells ± SD of five individual mice are shown.

booster injections were given. Spleen cells obtained from immunized mice 12–15 days postvaccination were restimulated ex vivo for 5 h with antigenic peptides. The fraction of the splenic $CD8^+$ T cells specifically inducible to IFN-γ expression by stimulation with the respective epitope was determined by FCM. In B6 mice, readily detectable $CD8^+$ T cell responses against the D^b-restricted influenza epitope 1, the K^b-restricted OVA epitope 2, the D^b-restricted adenovirus epitope 6, and the K^b-restricted Sendai virus epitope 7 were efficiently coprimed. As expected, H-2^b mice did not generate $CD8^+$ T cell responses against epitopes binding to H-2^d class I molecules (Fig. 2*A*). Unexpectedly, priming of $CD8^+$ T cell responses to the K^d-restricted influenza epitope 3 and the L^d-restricted CMV epitope 5 was low and inefficient in BALB/c mice (Fig. 2*B*). Only priming to the K^d-restricted malaria epitope 8 and the L^d-restricted lymphocytic choriomengitis virus epitope 10 was reproducibly detected (Fig. 2*B*). The pCMV/pt10 DNA vaccine thus efficiently primes $CD8^+$ T cell responses in H-2^b mice, but seems to be deficient in stimulating $CD8^+$ T cell responses in H-2^d mice.

L^d-restricted CTL down-modulate $CD8^+$ T cell priming to other epitopes presented by different MHC class I molecules

We have reported that $CD8^+$ T cell primed to the immunodominant, L^d-restricted HBsAg epitope suppresses copriming of $CD8^+$ T cells to simultaneously presented K^d-, D^d-, and K^b-restricted HBsAg epitopes (17). We therefore tested whether the pt10 DNA vaccine can elicit $CD8^+$ T cell responses to K^d-restricted epitopes 3 and 8 in L^{d-} BALB/c substrain dm2 mice. Priming of $CD8^+$ T cell precursors to these two epitopes was strikingly enhanced in dm2 mice (i.e., under conditions where no L^d-restricted $CD8^+$ T cell epitopes were presented; Fig. 2, compare *C* with *B*). Hence, priming L^d-restricted $CD8^+$ T cells seems to down-regulate priming of $CD8^+$ T cells to other epitopes of the construct, apparently regardless of their restriction or the nature of the epitope.

We immunized F1 (B6 × BALB/c) mice (Fig. 2*D*) to test whether the pt10 DNA vaccine can coprime multispecific, H-2^b- and H-2^d-restricted $CD8^+$ T cell responses. Compared with vaccinated B6 mice, the H-2^b-restricted $CD8^+$ T cell responses to epitopes 1, 2, 6, and 7 were deficient in F_1 (B6 × BALB/c) mice (Fig. 2, *A* and *D*). The H-2^d-restricted $CD8^+$ T cell responses against epitopes 3, 5, 8, and 10 primed in BALB/c and F_1 (B6 × BALB/c) mice were comparable (Fig. 2, *B* and *D*). When L^{d-} F_1 (B6 × dm2) mice were used instead of F_1 (B6 × BALB/c) mice, efficient priming of $CD8^+$ T cell responses to H-2^b-restricted epitopes 1, 2, 6, and 7 and K^d-restricted epitopes 3 and 8 was observed (Fig. 2*E*). These data indicate that 1) the pt10 DNA vaccine can efficiently coprime multispecific $CD8^+$ T cell responses restricted by at least four different MHC class I molecules; and 2) priming of L^d-restricted $CD8^+$ T cell responses can down-regulate copriming of $CD8^+$ T cells to epitopes restricted by unrelated MHC class I molecules.

L^d-restricted $CD8^+$ T cell responses, not Ld surface expression, down-modulate copriming of $CD8^+$ T cells restricted by unrelated MHC class I molecules

The K^b-restricted, OVA epitope 2 (SIINFEKL)-specific $CD8^+$ T cell responses were efficiently elicited by the pCMV/OVA DNA vaccine (encoding the complete OVA sequence) in B6 mice, F_1 (B6 × dm2) mice, and F_1 (B6 × BALB/c) mice (Fig. 3*A*; groups

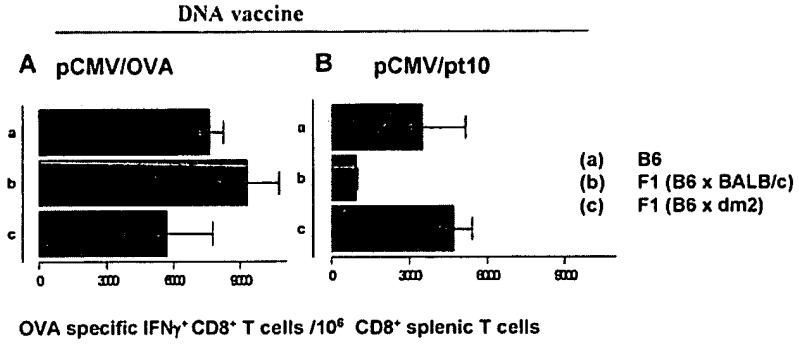

FIGURE 3. Priming OVA-specific $CD8^+$ T cell responses. B6 (*a*), F_1 (B6 × BALB/c) (*b*), and F_1 (B6 × dm2) (*c*) mice were vaccinated i.m. with 100 μg of OVA-encoding pCMV/OVA (*A*) or the polytope-encoding pCMV/pt10 (*B*) plasmid DNA. Spleen cells obtained 14 days postvaccination were restimulated for 5 h with H-2^b-restricted OVA epitope 2. T cells were surface-stained for CD8 and intracellularly stained for IFN-γ. We determined the frequencies of $CD8^+$ IFN-γ^+ $CD8^+$ T cell per 10^6 $CD8^+$ spleen cells by FCM analyses. The mean numbers of IFN-γ^+ $CD8^+$ T cells per 10^6 $CD8^+$ spleen cells ± SD of six individual mice of two experiments are shown.

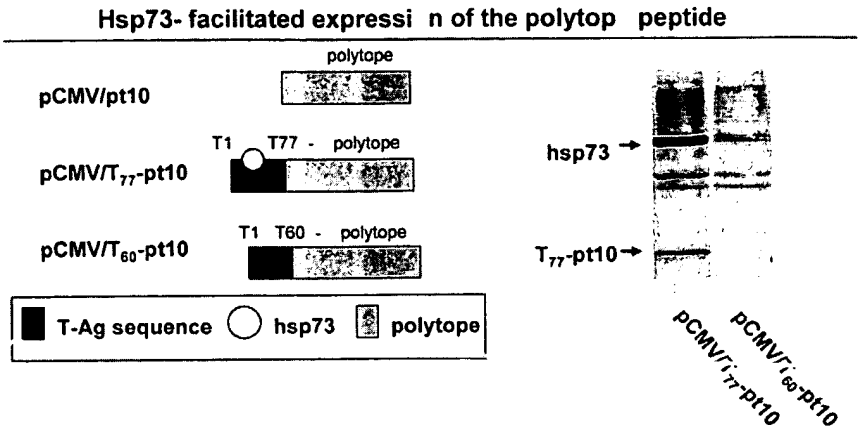

FIGURE 4. Expression of the 106-residue pt10 unit using the hsp73-binding expression system. Maps of pt10 and its fusion to the hsp73-binding T_{77} and non-hsp-binding T_{60} fragments are shown. LMH cells were transiently transfected with pCMV/T_{77}-pt10 or pCMV/T_{60}-pt10, labeled with [^{35}S]methionine, extracted and immunoprecipitated with anti T-Ag mAb 108, and processed for SDS-PAGE, followed by fluorography of the gels. The positions of hsp73 and the T_{77}-pt10 Ag are indicated.

a–c). The pt10 DNA vaccine contains this K^b-restricted OVA epitope 2. $CD8^+$ T cells specific for this epitope were efficiently primed by the pt10-encoding DNA vaccine in B6 and F_1 (B6 × dm2) mice, but not in F_1 (B6 × BALB/c) mice (Figs. 2 and 3*B*). Hence, copriming L^d-restricted $CD8^+$ T cell responses against epitopes encoded by pt10 (see Fig. 2), but not the surface expression of L^d, down-modulates copriming of $CD8^+$ T cell to other epitopes restricted by unrelated MHC class I molecules and present on the same antigenic construct.

Construction of an hsp73-associated polytope DNA vaccine

The pt10 vaccine used primed some $CD8^+$ T cell responses inefficiently. We tested whether alternative designs of the pt10 DNA vaccine enhance the efficiency of multispecific $CD8^+$ T cells priming by this construct and can overcome the suppressive effect of L^d-restricted $CD8^+$ T cell priming. We used an hsp73-mediated expression system (14, 15) by fusing the hsp73 binding SV40 T-Ag-derived DnaJ domain N-terminally in-frame to the polytope-encoding DNA sequence. The 106-residue-encoding pt10 sequence was fused C-terminally to the hsp73-binding, 77-residue, N-terminal T-Ag fragment, to generate the cT_{77}-pt10 chimeric protein (Fig. 4). As a negative control, the T_{60} N terminus of the T-Ag that does not bind hsp73 (14, 15) was fused to the pt10 sequence to generate the non-hsp-binding fusion protein T_{60}-pt10 (Fig. 4). The expression of the fusion proteins from the expression constructs pCMV/T_{77}-pt10 and pCMV/T_{60}-pt10 was tested after transient transfection of LMH (chicken hepatoma) cells. The mAb 108 (directed against the extreme N terminus of the T-Ag) was used for immunoprecipitation of chimeric Ags from the lysates of the transfectants. Subsequent SDS-PAGE analyses revealed efficient expression of the T_{77}-pt10 fusion protein (Fig. 4). We barely detected the non-hsp-associated T_{60}-pt10 fusion protein (Fig. 4), confirming our observation that stress protein-associated expression of the peptide was always higher than the expression of the corresponding non-hsp73-associated, chimeric proteins (14, 15). The hsp73 stress protein was coprecipitated with the fusion protein from transfected cells expressing peptides encoded by pCMV/T_{77}-pt10, but not from transfected cells expressing peptide encoded by pCMV/T_{60}-pt10 (Fig. 4). This confirms that the tight, noncovalent association between hsp73 and the viral DnaJ domain requires the intact 77-residue domain as described by us in other Ag systems (10, 11, 13, 15, 16). Hence, we have constructed DNA vaccines carrying the immunogenic pt10 domain, but differing in the level of expression and the association with hsp73.

Hsp-associated expression of polytope vaccines facilitates copriming of multispecific $CD8^+$ T cell responses

F_1 (B6 × BALB/c) mice were immunized by a single i.m. injection of the DNA vaccines pCMV/pt10 (Fig. 5; group a), pCMV/T_{77}-pt10 (group b), and pCMV/T_{60}-pt10 (group c). The primed $CD8^+$ T cell responses were read out against the H-2^b-restricted, pt10-encoded epitopes 1, 2, 6, and 7 (Fig. 5*A*) and against the H-2^d-restricted epitopes 3, 5, 8, and 10 (Fig. 5*B*). The hsp73-associated expression of the polytope DNA vaccine (group b) was reproducibly superior to the non-hsp-associated expression of the same DNA vaccine (group c) in priming all multispecific $CD8^+$ T

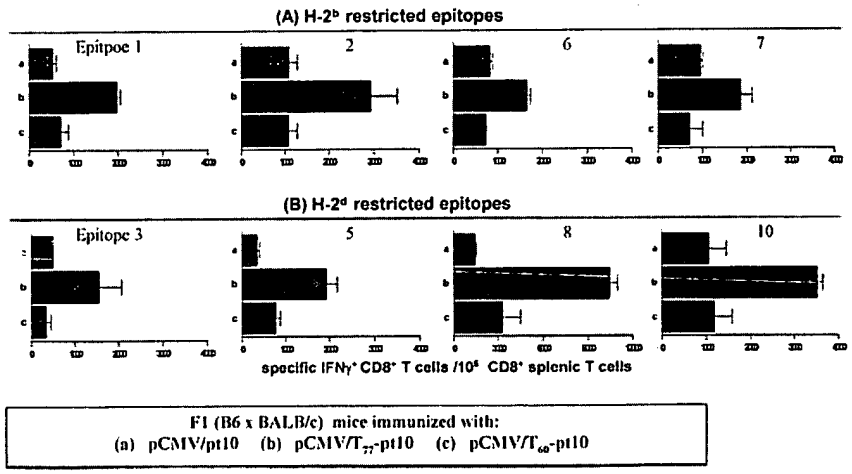

FIGURE 5. Hsp73-mediated priming of pt10-specific $CD8^+$ T cell responses. F_1 (B6 × BALB/c) mice were vaccinated i.m. with 100 μg of plasmid DNA encoding the pt10 polypeptide (*a*), the hsp73-associated T_{77}-pt10 chimeric protein (*b*), or the T_{60}-pt10 chimeric protein (*c*). Spleen cells obtained 12 days postvaccination were restimulated for 5 h with H-2^b-restricted epitopes 1, 2, 6, and 7 (*A*) and with H-2^d restricted epitopes 3, 5, 8, and 10 (*B*). T cells were surface-stained for CD8 and intracellularly stained for IFN-γ. We determined the frequencies of $CD8^+$ IFN-γ^+ $CD8^+$ T cell per 10^6 $CD8^+$ spleen cells by FCM analyses. The mean numbers of IFN-γ^+ $CD8^+$ T cells per 10^6 $CD8^+$ spleen cells ± SD of four individual mice are shown.

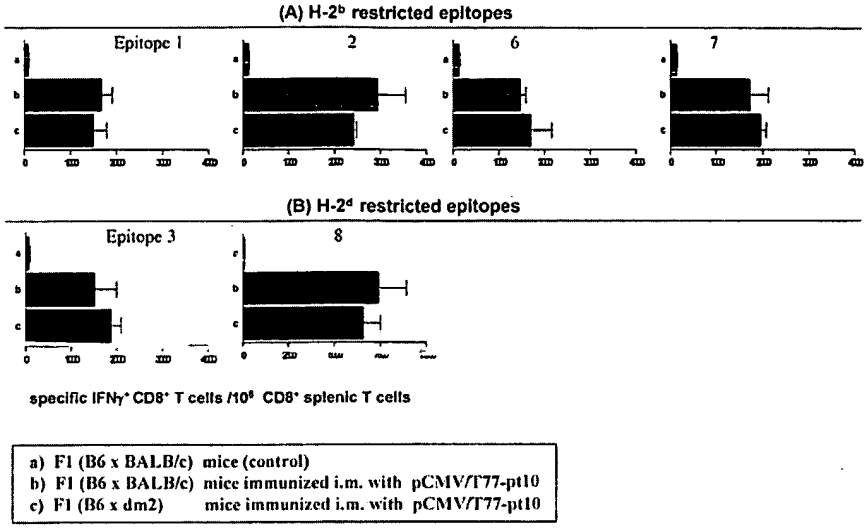

FIGURE 6. The hsp73-bound T_{77}-pt10 vaccine overrides suppression of coprimed, L^d-restricted $CD8^+$ T cell responses. F_1 (B6 × BALB/c) mice either were not vaccinated (*a*) or were vaccinated i.m. with 100 μg of plasmid DNA encoding the T_{77}-pt10 expression construct (*b*). F_1 (B6 × dm2) mice were immunized i.m. with 100 μg of plasmid DNA encoding the T_{77}-pt10 expression construct (*c*). Spleen cells obtained 12 days postvaccination were restimulated for 5 h with the H-2^b-restricted epitopes 1, 2, 6, and 7 (*A*) or the H-2^d-restricted epitopes 3, 5, 8, and 10 (*B*). T cells were surface-stained for CD8 and intracellularly stained for IFN-γ. We determined the frequencies of $CD8^+$ IFN-γ^+ $CD8^+$ T cell per 10^6 $CD8^+$ spleen cells by FCM analyses. The mean numbers of IFN-γ^+ $CD8^+$ T cells per 10^6 $CD8^+$ spleen cells ± SD of three individual mice are shown.

cell responses tested. No reproducible differences in the relative efficacy of $CD8^+$ T cell priming were detectable between vaccinations with the conventional pt10 vaccine (group a) and the pCMV/T_{60}-pt10 vaccine (group c). The expression of antigenic domains in tight, noncovalent association with hsp73 thus enhances their immunogenicity for $CD8^+$ T cells. Comparable $CD8^+$ T cell responses to the different epitopes were primed in F_1 (B6 × BALB/c) and F_1 (B6 × dm2) mice (Fig. 6, compare groups b and c). Thus, priming to L^d-restricted epitopes by the pt10 vaccine, but not the T_{77}-pt10 vaccine, down-modulated copriming to other $CD8^+$ T cell responses.

The efficacy of hsp73-binding T_{77}-pt10 Ag to specifically prime pt10-specific $CD8^+$ T cell precursors was even more striking in gene gun-mediated, intradermal DNA vaccination (Fig. 7). While priming of IFN-γ-producing, pt10-specific $CD8^+$ T cell responses was not detectable after injection of DNA vaccines encoding the non-hsp-binding pt10 (group a) or T_{60}-pt10 construct (group c), they were readily detectable after intradermal injection of the hsp73-binding T_{77}-pt10-encoding plasmid DNA with the gene gun (group b). The association of Ag with hsp73 thus strikingly enhances its immunogenicity for $CD8^+$ T cell precursors, supporting $CD8^+$ T cell priming even after intradermal delivery of low doses of DNA vaccines. Hence, a high level of Ag expression in association with hsp73 efficiently induces pt10-specific $CD8^+$ T cell responses even under L^d-dependent immunodominance, thereby facilitating copriming of $CD8^+$ T cell populations with a diverse repertoire.

Discussion

For this study we constructed DNA vaccines that express the polytope sequence pt10. This chimeric Ag was expressed in a form that either stably bound the constitutively expressed, cytosolic hsp73 molecule or was not hsp bound. As every nascent protein emerging from the ribosome transiently binds hsp73, it seems that the DnaJ-homologous sequence in the nascent polytope protein delays the off rate of hsp. As the DnaJ homologous sequence is present in the N terminus of native as well as mutant T-Ag, and only mutant, not native, T-Ag shows stable hsp73 binding, additional downstream signals (such as accessible epitopes bound by hsp73 substrate binding sites) seem to be required for stable hsp binding. Alternatively, specific post-translational capture of the chimeric, cytosolic protein by hsp73 to target it for alternative, endolysosomal degradation may operate in this system (43–45). The long half-life of the hsp73/protein complexes may reflect their delayed clearance from the cytosol and/or their extended survival in an endolysosomal compartment. The data in Fig. 4 confirm that stable hsp73

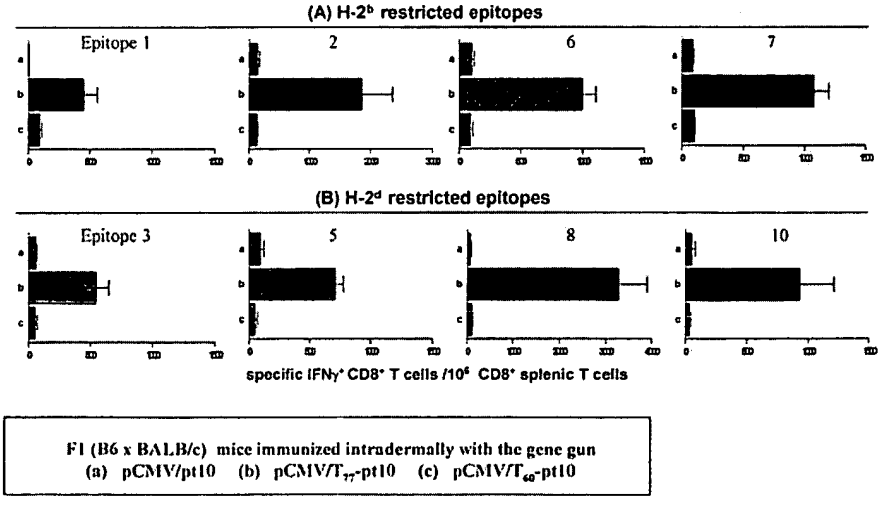

FIGURE 7. Hsp73-mediated priming of pt10-specific $CD8^+$ T cell responses using the gene gun delivery method. F_1 (B6 × BALB/c) mice were vaccinated and boosted intradermally (using the gene gun) with 2 μg of plasmid DNA of the indicated vectors: pCMV/pt10 (*a*), pCMV/T_{77}-pt10 (*b*), and pCMV/T_{60}-pt10 (*c*). Spleen cells obtained 12 days after the booster injection were restimulated for 5 h with the H-2^b-restricted epitopes 1, 2, 6, and 7 (*A*) or the H-2^d-restricted epitopes 3, 5, 8, and 10 (*B*). T cells were surface-stained for CD8 and intracellularly stained for IFN-γ. We determined the frequencies of $CD8^+$ IFN-γ^+ $CD8^+$ T cell per 10^6 $CD8^+$ spleen cells by FCM analyses. The mean numbers of IFN-γ^+ $CD8^+$ T cells per 10^6 $CD8^+$ spleen cells ± SD of three to six individual mice are shown.

binding allows the expression of protein fragments, which difficult to achieve without hsp binding (12, 15). This offers the chance to express selected immunogenic domains from different protein Ags of interest. The limits of the carrying capacity of the system are not yet clear, but up to 800 residues have been successfully expressed in the system (R. Schirmbeck, manuscript in preparation). This would allow the incorporation of long spacer regions (to block generation of junctional epitopes) (4) or long flanking regions (to facilitate natural processing of the epitope) into a construct. Hence, the system may overcome some of the restrictions in size and heterogeneity inherent in conventional peptide-based approaches.

The use of the chimeric Ag certainly is not restricted to DNA vaccination, but preparative isolation of hsp73/chimeric protein complexes is a feasible option, as has been expertly demonstrated for hsp/peptide complexes (33, 46–48). In addition to facilitating expression, hsp binding offers other features that make it attractive as a vaccine. Hsp molecules of the hsp70 and hsp90 class are intrinsic adjuvants (as indicated above) and introduce cytosolic proteins into alternative processing pathways (10, 11). As different proteolytic systems seem to give rise to different, although overlapping, repertoires of antigenic epitopes (49), this means of expression can potentially extend the repertoire of immunogenic epitopes presented by an Ag.

We and others have described the immunodominance of the L^d-restricted $CD8^+$ T cell response that interferes with the coprimring of $CD8^+$ T cells restricted by other MHC class I molecules in the HBsAg system (17, 50). These data are extended in this report. Two additional L^d-restricted epitopes from two unrelated viruses, i.e., murine CMV and lymphocytic choriomengitis virus, showed a similar immunodominance. This implies either that immunodominance is a property of L^d-restricted $CD8^+$ T cell responses or that we detected by chance three independent examples of immunodominant L^d-restricted $CD8^+$ T cell responses. Detailed analysis of L^d-restricted $CD8^+$ T cell responses in other systems may provide an answer. The cellular and molecular mechanisms that mediates this suppression are unknown, but are under active study in our group. It can be overcome at least partially by supplying high doses of type I IFNs and/or IL-15 to the site where $CD8^+$ T cell precursors are primed in situ (51). This suggests the involvement of a factor-mediated immunoregulation. Further studies are needed to elucidate this phenomenon.

We have previously described and reiterated above the potential advantages of delivery of hsp-bound Ag as vaccines. These include enhanced expression and facilitated expression of even large chimeric proteins (when used as a DNA vaccine), its facilitation of cross-priming, and its ability to introduce Ags into alternative processing pathways. The expression of the polytope vaccine as hsp-binding complexes not only enhanced its immunogenicity, but at least partially overcame the suppressive effect of L^d-dependent immunodominance. This may be related to the adjuvant effect of hsp, e.g., its cytokine-releasing effect. Taken together, these data support the idea that hsp-bound, large protein Ags produced in situ by DNA vaccines display exceptional immunogenicity and can escape suppressive immunoregulation.

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ATTACHMENT D

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Priming polyvalent immunity by DNA vaccines expressing chimeric antigens with a stress protein-capturing, viral J-domain

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ABSTRACT

The N-terminal domain of large tumor antigens (T-Ag) of polyomaviruses forms a DnaJ-like structure with a conserved J domain that associates with constitutively expressed stress protein heat shock protein (hsp)73. Mutant (but not wild-type) SV40 T-Ag show stable, ATP-dependent binding to the stress protein hsp73 when expressed in cells from different vertebrate tissues. Intracellular T/hsp73 complexes accumulate to high steady-state levels. From this observation, we designed a vector system that supports stable expression of a large variety of hsp73-capturing, chimeric antigens containing an N-terminal, T-Ag-derived domain, and different C-terminal antigenic domains from unrelated antigens. Most antigenic domains tested could be stably expressed only in eukaryotic cells as fusion protein/hsp73 complexes. The N-terminal 77 residues representing the J domain of T-Ag were required for stable hsp73 binding and efficient expression of chimeric antigens. Hsp73-bound chimeric antigens expressed by DNA vaccines showed strikingly enhanced immunogenicity evident in humoral (antibody) and cellular cytolytic T lymphocytes (CTL) responses. The described system supports efficient expression of chimeric, polyvalent antigens and their codelivery with hsp73 as a "natural adjuvant" for enhanced immunogenicity for T and B cells.

Key words: stress protein • antigen expression • DNA-based vaccines

The expression of isolated protein domains, mutant or truncated proteins, or selected antigenic epitopes of proteins is difficult but would greatly facilitate functional or immunological studies of complex proteins. Expression of selected amino acid sequences as fusion proteins is often chosen to produce detectable amounts of the protein fragment of interest. Molecular chaperones, in particular heat shock proteins (hsp), assist in protein folding, degradation, and traffic (see ref 1 for a review). Some members of the hsp70 family, for example, the abundantly and constitutively expressed hsp73 (hsc70) in the cytosol of mammalian cells, can stabilize mutant proteins but also facilitate their elimination in a novel lysosomal degradation pathway for intracellular proteins (2, 3). Hsp 73-associated expression seems an attractive option to enhance the level of fusion protein produced by transfectants.

The N-terminus of the large tumor antigens (T-Ag) of papovaviruses (SV40, polyoma virus) contains the J domain (i.e., sequences homologous to the *Escherichia coli* DnaJ molecule) with a conserved HPD loop that recruits cellular chaperones, for example, the cytosolic hsp73 chaperone (4, 5). These viral J domains are functional and stimulate ATPase activity of hsp73 (4). The association of the SV40 T-Ag with cellular hsp73 is facilitated by mutations and truncations of this nucleoprotein that leave the N-terminus intact. We have demonstrated tight binding of hsp73 to different variants of the T-Ag but not to wild-type (wt) T-Ag (6, 7). The large amount and long half-life of mutant, hsp73-associated T-Ag expressed by transfectants was unexpected. Similarly, fusion constructs containing an N-terminal hsp73-binding domain of T-Ag and unrelated C-terminal sequences encoding fragments of proteins showed strikingly enhanced and stable expression (8). This novel expression system allowed us to efficiently and selectively express antigenic epitopes of interest.

Hsp molecules are "innate adjuvants" that can enhance and modulate the immunogenicity of vaccines. Three different approaches have exploited the adjuvant effect of hsp molecules of the hsp70 and hsp90 family. When antigenic peptides are noncovalently "loaded" (*in vitro* or *in vivo)* to purified hsp molecules and injected into mice, they display greatly enhanced immunogenicity (9–11). Similarly, antigenic protein determinants fused to hsp molecules and injected as recombinant fusion proteins efficiently elicit $CD4^+$ helper T-cell-independent $CD8^+$ T-cell responses (12–16). Our approach involved the expression of CTL epitopes as chimeric fusion proteins containing a hsp73-binding, N-terminal viral J domain (8). This allowed high-level antigen expression in noncovalent but tight association with constitutively expressed, cytosolic hsp73 molecules. Here, we describe binding of hsp73 to chimeric antigens and characterize the immunogenicity of hsp73-associated antigens (vs. nonassociated antigens) delivered by plasmid DNA-based vaccines.

MATERIALS AND METHODS

Mice

$H\text{-}2^b$ C57BL/6JBom (B6) mice and $H\text{-}2^d$ BALB/cJBom mice were bred and kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). Male female mice were used at 12–16 wk of age.

Cells

The $H\text{-}2^d$ mastocytoma cell line P815 (TIB64) and the chinese hamster ovary (CHO) cell line (CRL-1772) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). The $H\text{-}2^b$ (B6-derived) T lymphoma cell line RBL5 was obtained from Dr. H.-U. Weltzien (Freiburg, Germany). RMA-S cells were a generous gift of Dr. K. Kärre (Stockholm, Sweden). The BALB/c-derived fibrosarcoma Meth-A cell line were kindly provided by Dr. W. Deppert (Hamburg, Germany). The chicken hepatoma cell line LMH was obtained from Dr. H-J. Schlicht (Ulm, Germany).

Vector constructs

We used the BMG*neo* vector system for the generation of stable transfectants in which genes are expressed under methallothionin promotor control (17). The wt T-Ag of SV40, the mutant cytoplasmic cT-Ag variant (with a deletion of the SV40 nucleotide sequence 4490-4392, representing the nuclear localization signal (NLS)-encoding amino acid sequence $T_{110-152}$ or the N-terminal T_{272} fragment were cloned into the BMG*neo* vector as described previously (18). The BMG/$T_{411-708}$ construct was generated from the T-Ag encoding plasmid pEARLY (19). The $T_{411-708}$ encoding NsiI/BamHI fragment of pEARLY was cloned into the PstI/BamHI site of pBluescript (cat. no. 212205; Stratagene-Europe, Amsterdam). The resulting plasmid pBlue/$T_{411-708}$ was cut with XhoI and BamHI and cloned into the XhoI/BamHI site of BMG*neo*, thus generating the plasmid BMG/$T_{411-708}$. The vector BMG/cT_{272}-RT132 was generated from a cT-Ag encoding plasmid cT272-Blue and a 400-bp EcoRI-fragment encoding the aa sequence 281-412 of SIVmac 239 (a generous gift of Drs. H. Petry, Göttingen, and K. Melber, Düsseldorf). The EcoRI-fragment was cloned into the EcoRI site of pBluescript, generating the plasmid RT132-BlueA. The RT132-encoding HindIII/BamHI fragment of RT132-BlueA was cloned into the HindIII/BclII linerarized plasmid backbone of cT272-Blue. The resulting plasmid RT132-BlueB was cut with HindIII and fused with a cT272- encoding HindIII-fragment, generating the plasmid cT272-RT132-Blue. The cT272-RT132-Blue was cut with XhoI/BamHI and cloned into XhoI/BamHI cut BMGneo to generate the plasmid BMG/cT272-RT132 that encodes a fusion protein containing the SV40 cT272, a 4 aa spacer (DIEF), and the SIVpol 281-412 aa sequence. The construction of the plasmid BMGneo/C (encoding the hepatitis B core antigen HBcAg 1-183 aa) was described previously (20).

For transient transfection assays and plasmid DNA vaccination experiments, we used the vector pCI (cat. no. E1731; Promega, Mannheim, Germany) in which antigens are expressed under human cytomegalovirus promotor/enhancer control. Construction of the pCI vectors encoding the SV40 wt T, cT, T_{272}, or HBcAg has been described (6–8, 20).

Construction of the pCI/T_{272}-RT132 and pCI/cT_{272}-RT132 plasmids

The plasmid RT132-BlueB was cut with HindIII and fused to the T_{272}- or cT_{272}-encoding HindIII-fragments generating the plasmids T_{272}-RT132Blue or cT_{272}-RT132Blue. The Xho/BamHI fragments were cloned into pCI vector to generate the pCI/T_{272}-RT132 or pCI/cT_{272}-RT132 plasmids.

Construction of the pCI/T_{60}-C70 and pCI/T_{77}-C70 plasmids

The T_{60} fragment was amplified by polymer chain reaction (PCR) from the pCI/cT_{272}-RT132 plasmid, using a forward primer with a XhoI site (AAACTCGAGATGGATAAAGTTTTAAAC AGAGAGG) and a reverse primer with a HindIII site (AAAAAGCTTCTTGTACAGAG TATTCATTTTCT TC). The product was cloned into pBluescript to generate the plasmid pBlue/T_{60}. The T_{77} fragment was amplified by PCR, using the same forward primer with a XhoI site (AAACTCGAGATGGATAAAGTT TTAAACAGAGAGG) and a reverse primer with HindIII site (AAAAAGCTTGAAGCCTCCAA AGTCAGGTTG). This product was cloned into pBluescript to generate the plasmid pBlue/T_{77}. The HBcAg sequnce encoding residue 79-149 was amplified with PCR, using the primers GGAAGCTTCCAGC GTCTAGAGACCTAGTA

and GGGCGGCCGCCTAAACAACAGTAG TCTCCGGAAG introducing HindIII and NotI restriction sites and stop signals after HBcAg position 149. The PCR product was cloned in frame into pBlue/T_{60} and pBlue/T_{77} digested with HindIII/NotI. The fusion protein-encoding sequences T_{60}-C70 and T_{77}-C70 were cloned into the pCI vector, using XhoI/NotI restriction enzymes to create the pCI/T_{60}-C70 and pCI/T_{77}-C70 vectors.

Construction of the pCI/T_{60}-preS163 and pCI/T_{77}-preS163 plasmids

The preS (S1/S2) antigens of HBVayw were PCR-amplified using the sense primer AAAAAGCTTATGGGGCAGAATCTTTCCACCAGC and the antisense primer AAAGGTACC GTTCAGCGCAGGGTCCCCAATCC. These sequences were inserted behind the pCI/T_{60} or the pCI/T_{77} as described previously.

Construction of HBV-S fragment encoding vectors

pCI/cT_{272}-SI-III: The HBV-sequences encoding the HBV-S sequence from 1-100, 80-180, or 140-226 were cloned in frame to the hsp73-binding cT-Ag_{272} fragment, using CCCAAGCTTATGGAGAACATCACATCAGGA (+) and GGAAAAAAGCGGCCGCTTAA TAGTCCAGAAGAACCAACAA (-); CCCAAGCTTATCATCTTCCTCTTCATCCTGCTG (+) and GGAAAAAAGCGGCCGCTTAAACAAATGGCACTAGTAAACT (-); or CCCAAGCTTAA ACTTTCGGACGGAAATTGC (+) and GGAAAAAAGCGGCCGCTTAAATGTATACCCA AAGACAAAAG (-) primers. pCI/T77-SIII.1-3: The HBV-sequences, encoding the HBV-S sequence 140-175, 167-205, or 195-226 were cloned in frame to the hsp73-binding T77 fragment, using the CCCAAGCTTAAACCTTCGGACGGAAATTGC (+) and AAATCTAGATTATAAACTGAGCCAGGAGAAAACG (-); CCCAAGCTTTCA GCCCGTTTCTCC TGGC (+) and AAATCTAGATTACAGACTTGGCCCCCAATACC (-); or the CCCAAGCTTATATGGATGATGTTGTATTGGGG (+) and AAATCTAGATTAAATGTATA CCCAAAGACAAAAGAA (-) primers.

DNA vaccination

For intramuscular nucleic acid immunization, we injected 50 µl phosphate-buffered saline (PBS) containing 1 µg/µl plasmid DNA into each tibialis anterior muscle as described previously (8, 21). Intradermal injection of 1 µg particle-coated DNA with the Helios Gene Gun system (BioRad, Munich, Germany) has been described previously (22).

Transient and stable expression of fusion proteins

For transient expression, LMH cells were transfected with pCI-based plasmids, using the Ca_2PO_4 method. Cells were harvested 36 h after transfection, metabolically labeled for 12 h with [^{35}S]-methionine (Amersham, Braunschweig, Germany), and extracted with lysis buffer (120 mM NaCl, 1% aprotinin [Trasylol, Bayer, Leverkusen, Germany], leupeptin, 0.5% NP40, and 50 mM Tris-hydrochloride [pH 8.0]) for 30 min at 4°C. Extracts were cleared by centrifugation and immunoprecipitated for T-Ag by using the monoclonal antibody (mAb) PAb108 directed against the extreme N-terminus of the T-Ag (23) and protein A-sepharose. Immune complexes bound to

protein A sepharose were purified with wash buffer (300 mM LiCl, 1% NP40, and 100 mM Tris-hydrochloride [pH 8.5]), followed by two washes with PBS and 0.1× PBS. Immune complexes were recovered from protein A-sepharose with elution buffer (1.5% SDS, 5% mercaptoethanol, and 7 mM Tris-hydrochloride [pH 6.8]), processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analysed by fluorography. Western blot analyses were performed as described (6–8). Where indicated, hsp was precipitated from cell lysates with adenosine 5′ diphosphate cross-linked to agarose (Sigma, Taufkirchen, Germany).

Determination of splenic CTL frequencies

Spleen cells (1×10^7/ml) were incubated for 1 h in RPMI medium with syngenic HBcAg-expressing target cells (10^6/ml). Thereafter, 5 μg/ml brefeldin A (BFA) (Sigma) was added, and the cultures were incubated for an additional 4 h. Cells were harvested and surface stained with phycoerythrin (PE)-conjugated anti-CD8 mAb (BD-PharMingen, Heidelberg, Germany). Surface-stained cells were fixed with 2% paraformaldehyde in PBS before intracellular staining for IFNγ. Fixed cells were resuspended in permeabilization buffer (containing 0.5% bovine serum albumin [BSA], 0.5% saponin, and 0.05% sodium azide) and were incubated with fluoresecin isothiocyanate (FITC)-conjugated anti-IFNγ mAb (Pharmingen) for 30 min at 20°C and washed twice in permeabilization buffer. Stained cells were resuspended in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. The frequencies of peptide-induced $CD8^+$ $IFN\gamma^+$ CTL were determined by flow cytometry (FCM) analyses. The mean numbers of $CD8^+$ $IFN\gamma^+$ T cells/10^5 $CD8^+$ spleen cells of three to five individual mice are shown in the respective figures .

Cytotoxicity assays

Single-cell suspensions were prepared from spleens of mice in minimum essential medium α (α-MEM) tissue culture medium supplemented with 10 mM HEPES buffer, 5×10^{-5} M 2-ME, antibiotics, and 10% v/v fetal calf serum (FCS) (PAA Laboratories, Linz, Austria). A selected batch of Con A-stimulated rat spleen cell supernatant (2% v/v) was added to the culture medium. Responder cells (3×10^7) were cocultured with 1×10^6 irradiated, syngeneic transfectants. Coculture was performed in 10 ml medium in upright 25-cm^2 tissue culture flasks in a humidified atmosphere/5% CO_2 at 37°C. After 5 days of culture, CTL were harvested, washed, and assayed for specific cytolytic reactivity. Serial dilutions of effector cells were cultured with 2×10^3 ^{51}Cr-labeled targets in 200 μl round-bottom wells. Specific cytolytic activity of cells was tested in short-term ^{51}Cr-release assays against transfected, HBcAg-expressing targets or control targets. After a 4 h incubation at 37°C, 50 μl of supernatant were collected for γ-radiation counting. The percentage specific release was calculated as the following: [(experimental release – spontaneous release)/(total release – spontaneous release)] × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always <15% of the total counts. Data shown are the mean of triplicate cultures. The SE of triplicate data was always <20% of the mean.

Determination of serum antibody levels

Serum samples were repeatedly obtained from individual, immunized mice by tail bleedings.

Serum IgG, IgG1, and IgG2a antibodies against hepatitis B virus (HBV) core antigens were determined by end-point dilution ELISA assay as described previously (24). In brief, micro-ELISA plates (Nunc-Maxisorp, Nunc, Wiesbaden, Germany) were coated with 150 ng recombinant HBcAg or HBeAg per well in 50 µl 0.1 M sodium carbonate buffer, pH 9.5, at 4°C. Serial dilutions of the sera in loading buffer (PBS supplemented with 3% BSA, BSA, and 2% Tween 20) were added to the antigen-coated wells. Serum antibodies were incubated for 2 h at 37°C, followed by four washes with PBS supplemented with 0.05% Tween 20. Bound serum antibodies were detected using horseradish peroxidase-conjugated anti-mouse IgG antibodies (Pharmingen) at a dilution of 1:2000 followed by incubation with *o*-phenylendiamine ×2 HCl (Abbott, Wiesbaden, Germany) in PBS (pH 6.0). The reaction was stopped by 1 M H_2SO_4, and the extinction was determined at 492 nm. End-point titers were defined as the highest serum dilution that resulted in an absorbance value three times greater than that of negative control sera (derived form nonimmunized mice).

RESULTS

Hsp73 binds mutant SV40 T-Ag proteins with an intact N terminus

Cells transfected with pCI/T vector DNA express the nucleophilic, wt T-Ag of SV40 (Fig. 1A, 1B). Similar levels of expression were detected in cells transfected with pCI/cT vector DNA encoding mutant cytoplasmic cT-Ag from which residue 110-152 representing the NLS was deleted (Fig.1A, 1B). Hence, expression of mutant T-Ag protein was found to be unexpectedly stable. When wt T-Ag or cT-Ag was precipitated from lysates of stable transfectants with anti-T-Ag antibodies directed against N- (Pab108) or C-terminal (Pab101) domains, mutant cT-Ag, but not wt T-Ag, accumulated within cells in tight association with the constitutively expressed, cytoplasmic stress protein hsp73 (Fig. 1B; data not shown). ATP (but not ADP) released hsp73 from its complex with cT-Ag (Fig. 1C), demonstrating that the interaction of hsp73 and cT-Ag is ATP-dependent. Stable hsp-substrate complexes contained ADP-bound hsp (1). In this stabilized form, cT-Ag-hsp73 complexes were precipitable with ADP-agarose (Fig. 1C). Coprecipitation of hsp73 and cT-Ag by ADP agarose was confirmed by Western blot analyses (Fig. 1C). As expected, ADP-agarose also precipitated different hsp molecules bound with cellular polypeptides (Fig. 1C). The cT-Ag-hsp73 complexes were readily detected in lysates from transfectants obtained by different lysis methods, that is, comparable amounts of cT-Ag and hsp73 were immmunoprecipitated from cells lysed by NP40- or CHAPS-containing buffer or by detergent-free dounce homogenization (data not shown). Hence, cT-Ag/hsp73 complexes are surprisingly resistant to detergents. cT-Ag was efficiently bound to hsp73 in transfected cell lines of different species (human, mouse, monkey, chicken) and of different tissue origin (kidney, liver, skin, muscle) (data not shown). Therefore, mutant but not wt T-Ag forms stable complexes with hsp73 in cells from all vertebrate species and all tissues tested.

We did not detect an association between hsp73 and wt T-Ag in transiently and stable transfected cell lines using immunoprecipitation or Western analyses (Fig. 1B; data not shown). In pulse/chase analyses of T-Ag-expressing cells (using a 15-min pulse phase), we detected preferentially large polypeptides associated with hsp73 in anti-hsp73 immunoprecipitates (Fig. 2A). Hsp73 thus preferentially binds large polypeptides with high affinity (1). This interaction is transient as none of these proteins is detectable after a 60-min chase (Fig. 2A). Although the pulse/chase analyses provided no evidence for a stable association between hsp73 and wt T-Ag

(Fig. 2A), a stable cT-Ag/hsp73 association was evident throughout the entire chase period (during which all other cellular polypeptides dissociate from hsp73) (Fig. 2A). Hence, binding of hsp73 to cT-Ag differs from binding of hsp73 to most cellular proteins and to wt T-Ag.

Design of vectors expressing stress protein-capturing, chimeric antigens

We have developed a system for expressing chimeric antigens containing an hsp73-capturing, N-terminal T-Ag-derived domain and an antigenic domain of an unrelated virus protein (8). We fused the N-terminal, hsp73-binding T_{272} fragment in frame to a sequence of the SIVpol gene encoding 132 aa of the reverse transcriptase (RT) domain $pol_{281-412}$ (Fig. 3A). This RT domain could not be expressed by recombinant vector constructs encoding only the RT-encoding sequence (data not shown) but was readily expressed in transfectants from pCI/T_{272}-RT132 vector DNA as a chimeric protein (Fig. 3A, 3B). Immunoprecipitation analyses using anti-T-Ag or anti-hsp73 antibodies revealed stable association of hsp73 with the T_{272}-RT132 fusion protein in transiently and stably transfected cell lines (Fig. 3A, 3B; data not shown). The T_{272}-RT132 fusion protein captured more hsp73 than the T_{272} protein, suggesting that hsp73 also binds to the C-terminal polypeptide (Fig. 3B). The expression of the RT-containing fusion protein was improved two- to threefold when the NLS was deleted from the hsp73 binding T-Ag N-terminus to generate the cT_{272}-RT132 construct (Fig. 3A, 3C). The hsp73-associated cT_{272}-RT132 fusion protein accumulated to steady-state levels in transfected cell lines that were easily detectable in Coomassie Blue-stained gels (Fig. 3D). Western blot analyses confirmed that the fusion protein contained sequences derived from the T-Ag and the RT of SIV-RT, and that the 70-kDa protein that specifically coprecipitated with the fusion protein was hsp73 (Fig. 3D). Quantitative analyses of immunoprecipitates from stable transfectants indicated that 0.2–1 μg of cT_{272}-RT132 fusion proteins are expressed by 10^7 cells (Fig. 3E).

Minimal sequence of the T-Ag N-terminus required for hsp73 binding

We could stably express N-terminal T-Ag fragments (e.g., the T_{272} fragment) but not C-terminal T-Ag fragments (e.g., the $T_{411-708}$ fragment) in cell lines (Fig. 1A, 1B). The N-terminus of SV40 T-Ag contains the J domain homologous to the *E. coli* DnaJ protein (see refs 4 and 5 for reviews). The J domain of the T-Ag contains the conserved HPD sequence at the top of a loop between two α-helices (Fig. 2B) that is required for its interaction with hsp73. The J domain stimulates ATPase activity of hsp73 and facilitates docking of polypeptide substrates to this chaperone. To confirm the role of this DnaJ structure of the T-Ag N-terminus for hsp73 binding, we reduced it to 77 aa (i.e., the minimal sequence containing the intact T-Ag-specific DnaJ structure). We fused the antigenic sequence from the 163-residue HBV preS (preS1/preS2) domain in frame to the T_{77} fragment (Fig. 4A). We fused the 60-residue T_{60} fragment N-terminal to the C-terminal HBV preS domain (Fig. 4A). The helical loop structure of the J domain required for the contact of HPD with hsp73 is destroyed in T_{60} but not in the T_{77} fragment (Fig. 2B) (4). The anti-T-Ag mAb Pab108 immunoprecipitated the fusion proteins T_{60}-preS163 and T_{77}-preS163 from lysates of transfected cells. The hsp73 molecule was coprecipitated with the T_{77}-preS163 fusion protein but not the T_{60}-preS163 fusion protein (Fig. 4B). Furthermore, the hsp73-associated T_{77}-preS163 protein but not the T_{60}-preS163 protein accumulated in transfected cells to high steady-state levels readily detectable in Coomassie Blue-stained gels of immunoprecipitates (Fig. 4C). Western analyses confirmed that hsp73 was coprecipitated with

the T_{77}-preS163 but not the T_{60}-preS163 fusion protein (Fig. 4C). The preS domain could not be expressed by vector constructs encoding only the preS-encoding sequence (8) but low-level T_{60}-preS163 fusion protein expression was obtained. The N-terminal 77-residue sequence of T-Ag containing the intact J domain thus is required to efficiently express chimeric antigens in tight association with hsp73.

Hsp73-capturing, chimeric antigens display enhanced immunogenicity

We compared the immunogenicity of hsp73-capturing vs. non-hsp-associated chimeric antigens by using an antigenic fragment from the HBV core gene with well-defined antibody and CTL epitopes. The HBV core gene encodes the 183-residue HBcAg and the 159-residue HBeAg. Both proteins share a 149 aa sequence that contains the two nested, K^d- or K^b-binding CTL epitopes (20, 25) and two major antibody-binding epitopes, c/e1 and e2 (see ref. 26 for a review) (Fig. 5A). The 70-residue $HBcAg_{79-149}$ fragment C70 that contains the CTL motifs and the antibody-defined e2 determinant was cloned in frame behind either the non-hsp-binding T_{60} fragment or the hsp73-binding T_{77} fragment (Fig. 5A). Transient transfection analyses showed high expression of the chimeric, hsp73-binding T_{77}-C70 antigen, and low expression of the chimeric T_{60}-C70 antigen not associated with hsp (Fig. 5B) similar to the data described previously for the preS domain (see Fig. 4).

Both expression constructs were used as DNA vaccines by injecting i.m. 100 μg/mouse of these plasmid DNAs into BALB/c mice. Specific serum antibody and CTL responses were read out 3–8 wk postvaccination. Although the T_{60}-C70 and hsp73-associated T_{77}-C70 antigens were not secreted from transfected cells (data not shown), the pCI/T_{77}-C70 DNA immunizations stimulated the induction of high levels of HBcAg- specific serum antibodies (Fig. 5C). Titers of specific serum antibodies induced by the injection of the DNA vaccine encoding the hsp73-binding chimeric antigen were reproducibly 15- to 25-fold higher than the titers in the serum of mice injected with the DNA vaccine encoding the non-hsp-binding variant of the chimeric antigen. Very similar results were obtained when HBeAg was used as the detection antigen, but the antibody titers were reduced (data not shown). The e2-specific antibody response specifically stimulated by hsp73-binding chimeric T_{77}-C70 antigen was thus strikingly enhanced.

These findings were confirmed in Western blot analyses. Serum antibodies derived from pCI/T_{77}-C70- (but not from pCI/T_{60}-C70-) vaccinated mice efficiently bound linear HBcAg determinants, using denatured, recombinant HBcAg as detection antigen (data not shown). Antibodies binding T-Ag were readily apparent in Western analyses, using sera from pCI/cT-immunized mice and the hsp73-associated cT_{272}-preS fusion protein as detection antigen (8). No T-Ag-binding antibodies were primed by DNA vaccines encoding the T_{60}- and T_{77}-containing fusion constructs, and we did not detect serum antibodies binding the hsp73 autoantigen, confirming our previous data (8). The expression of the C_{79-149} fragment as a fusion protein by the pCI/T_{60}-C70 and pCI/T_{77}-C70 DNA vaccines thus selectively presents the e2 determinant of HBV core to the immune system but does not elicit antibody responses to the T-Ag-derived carrier or the hsp autoantigen.

HBcAg-specific CTL were primed in BALB/c mice by injecting plasmid DNA encoding the T_{77}-C70 or the T_{60}-C70 antigens. High frequencies of $CD8^+$ $IFN\gamma^+$ CTL specifically stimulated by

P815/C cells (but not P815/T or P815 cells) were detected *ex vivo* in mice immunized with the pCI/T_{77}-C70 DNA (Fig. 5D). The frequency of specific CTL elicited by the pCI/T_{77}-C70 encoding plasmid DNA was significantly higher than the CTL frequency elicited by injecting the non-hsp-associated pCI/T_{60}-C70 encoding plasmid DNA (Fig. 5D), and was similar to the CTL frequency elicited by injecting pCI/C plasmid DNA encoding HBcAg (Fig.5D).

This pattern of CTL induction was confirmed in cytolytic assays in which *in vivo* primed splenic T cells were cocultured *in vitro* for 5 days with HBcAg-expressing, syngeneic transfectants and tested for specific cytolytic reactivity, using antigen-expressing targets. The pCI/T_{77}-C70 but not the pCI/T_{60}-C70 encoding DNA vaccine efficiently induced HBcAg-specific CTL that lysed P815/C but not control P815 cells (Fig. 5E). The efficacy of hsp73-binding T_{77}-C70 antigen to specifically prime core-specific CTL precursors was even more striking in gene-gun mediated, intradermal DNA vaccination experiments (Fig. 5F). Although priming of IFNγ-producing $CD8^+$ CTL was not detectable after injection of DNA vaccines encoding either non-hsp-binding T_{60}-C70 antigen or native HBcAg, it was readily detectable after i.d. injection of the hsp73-binding T_{77}-C70 encoding plasmid DNA with the gene gun (Fig. 5F). The association of antigen with hsp73 thus strikingly enhances its immunogenicity for CTL precursors supporting CTL priming even after i.d. delivery of low doses of DNA vaccines.

Identifying CTL epitopes using chimeric, hsp73-bound antigen fragments

We cloned overlapping sequences of the 226-residue HBV surface (HBsAg; S) gene in frame behind the hsp73-binding cT272 or T77 N-terminus (Fig. 6A). Three overlapping S fragments were chosen: the N-terminal 100-residue fragment SI (aa 1-100), the central 100-residue fragment SII (aa 80-180), and the C-terminal 86-residue fragment SIII (aa 140-226). S-specific CTL were primed in C57BL/6 ($H\text{-}2^b$) mice by injecting plasmid DNA encoding the cT_{272}-SIII antigen. $CD8^+$ $IFN\gamma^+$ CTL specifically stimulated by S-expressing RBL5/S cells (but not RBL5 cells) were detected *ex vivo* in mice immunized with the pCI/cT_{272}-SIII DNA (Fig. 6B). We then constructed three overlapping S3 fragments: the 35-residue fragment SIII.1 (aa 140-175), the 38-residue fragment SIII.2 (aa 167-205), and the 31-residue fragment SIII.3 (aa 195-226). S-specific CTL were primed by the T_{77}-SIII.2 -encoding construct (Fig. 6B). Hence, the HBV-S region 167-205 contains a novel CTL domain. T-specific CTL were induced by cT272 (but not T77) fusion constructs (Fig. 6B), confirming our previous data (8).

Transfection of cells revealed efficient expression of hsp73-bound cT272- and T77-fusion proteins (data not shown). In contrast, expression of hsp non-binding T60-fusion constructs, encoding the respective antigen fragments, was barely detectable, and these constructs did not induce S-specific CTL responses (data not shown). Hence, hsp73-mediated expression of large and small protein fragments can be used to identify CTL domains within an antigen.

DISCUSSION

The interaction of DnaJ-like proteins with hsp70 molecules

Hsp70 molecules contain two functional domains: a nucleotide-binding N-terminal ATPase domain and a peptide (substrate)-binding C-terminal domain. The latter recognizes distinct

motifs within a substrate, for example, four to five hydrophobic (e.g., leu) residues flanked by basic residues (27). Hsp70 can discriminate folded and unfolded proteins because these motifs, found on average, on every 36 residues in all cellular proteins (27), preferentially in β-sheets, are not accessible for hsp70 recognition in native proteins. Immunoprecipitation analyses of pulse/chase labeled cells with anti-hsp73 antibodies showed that hsp73 preferentially binds to nascent, large but not short polypeptides (Fig. 2A). Small proteins fold more rapidly into native conformations and/or exhibit a low affinity for hsp73 as their number of potential hsp-binding motifs is reduced. Consistent with the proposed role of hsp73 in protein folding, we show that hsp73 associates only transiently with nascent cellular proteins (Fig. 2A).

Viral proteins containing J domains

The highly conserved J domain mediates the association of cellular DnaJ-like chaperones with hsp73 (28). The J domain is composed of four α-helices with helix II and III forming a finger-like structure that exposes the conserved HPD motif on the tip of the loop (4, 29). The N-terminal domain of polyomavirus T-Ag forms a DnaJ-like structure with the conserved J domain and the HPD sequence (Fig. 2B) that can associate with hsp73 (4, 30, 31). Mutations either in the HPD motif of the J-domain or in other sites of the 83-residue DnaJ-like N-terminus of the T-Ag disrupt the helical structure and prevent their association with hsp73 (Fig. 2B) (4). These mutants are defective in some T-Ag-dependent functions (e.g. viral DNA replication), suggesting that chaperone binding plays a key role in the viral life cycle (4).

T-Ag bound to hsp73 may target multiprotein complexes of the host cell that are involved in transcriptional regulation and/or viral DNA replication. We cloned the SV40 wt T-Ag sequence into expression vectors and analyzed transient and stable T-Ag expression. Stable expression of wt T-Ag in transfected cell lines was readily detected, but it was associated with only barely detectable (if any) binding to hsp73 molecules (6–8). Association of hsp73 with wt T-Ag may thus be limited to a small subpopulation of molecules and/or may occur only transiently. In contrast, many mutant T-Ags show stable association with hsp73 from different vertebrate species. The intact N-terminus of T-Ag containing the J domain is required for hsp73 association and stable expression of mutant T-Ag. We found that the T_{77} fragment, but not the T_{60} (with a disrupted DnaJ-homologous structure), binds to hsp73, confirming the key role of an intact DnaJ-like stucture with the conserved HPD motif for hsp73 association (4).

Mutant T-Ags associated with hsp73 have a surprisingly long half-life (>12 h) (6–8) and accumulate to steady-state levels of ~1 $\mu g/10^7$ cells. Hence, binding of hsp73 to mutant T-Ag differs from binding of hsp73 to most cellular proteins and to wt T-Ag. High ATP levels released hsp73 from T-fusion proteins (6, 7), suggesting that hsp73-bound fusion proteins accumulate in distinct cellular compartiments with low ATP levels. It is unknown why hsp73 interacts more strongly with the J domain of mutant T-Ag but not wt T-Ag, although all molecules contain the identical N-terminus. Our data suggest that the dissociation of hsp73-cT-Ag complexes is blocked, similar to the accumulation of stable complexes between Ig heavy-chain and the endoplasmatic reticulum (ER)-resident BiP chaperone (32). Our data also suggest that in addition to binding hsp73 through its DnaJ-like domain, mutant T-Ags may expose substrate motif(s) because they have a more unfolded conformation that bind the peptide-binding domain of hsp73, thereby locking the hsp/DnaJ structures.

Design of vector systems supporting hsp-facilitated expression of proteins

We designed a vector system for efficient expression of hsp73-associated, chimeric proteins in which an N-terminal, T-Ag-derived J-domain is fused to different C-terminal fragments of unrelated antigens. Many different sequences from heterologous viral antigens have been fused C-terminally to the T-Ag without negatively affecting the binding of hsp73 to the N-terminal T-Ag J domain. The observation that the expression system described can efficiently produce large chimeric protein antigens in stable association with hsp73 is of interest for vaccine designs. We designed vectors that allow expression of up to 800 residue chimeric proteins cloned behind the hsp73-binding DnaJ-like domain of T-Ag. Protein domains that could not be expressed without hsp73 association showed stable expression when fused to the J domain-containing N-terminus (e.g., the HBV preS domain) (Fig. 4). The system thus facilitates antigen expression by vector DNA.

Enhancing immunogenicity of vaccines by codelivery of antigen/hsp complexes

In addition to facilitating expression, hsp molecules enhance and modulate the immunogenicity of protein and peptide antigens (see refs 33 and 34 for reviews). Hsp molecules mediate their role as innate adjuvants (35–38) by binding to surface receptors (39, 40) such as CD91, TLR2/4, and/or CD14 (40–43). Hsp induce maturation of dendritic cells (DC) (14, 38, 44–47), trigger DC migration to lymph nodes, and stimulate their cytokine/chemokine release (38, 48). Associating antigen to hsp facilitates their processing for the generation of antigenic peptides binding to major histocompatibility complex (MHC) class II or class I molecules. As exogenous antigen/carrier complexes, hsp/peptide complexes deliver peptides for MHC class I-restricted processing/presentation, thereby facilitating priming of CTL to peptides from tumor antigens, minor H antigens, or viruses(10, 11, 49–55).

Hsp molecules support $CD4^+$ T helper-independent priming of CTL responses when delivered as an exogenous complex with antigens at low doses without additional adjuvants (13,14). We have shown that hsp73-bound endogenous antigen is submitted to TAP-independent, endolysosomal processing for MHC class I-restricted epitope presentation (6, 7), facilitates cross-priming of CTL (56) and priming of antibody responses to endogenous antigen by DNA vaccines (8). Here, we demonstrate the biochemical basis of these observations and confirm the broad range of potential applications for this expression system. The findings will be helpful for the rational design of CTL-stimulating vaccines against cancer and virus infections.

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Fig. 1

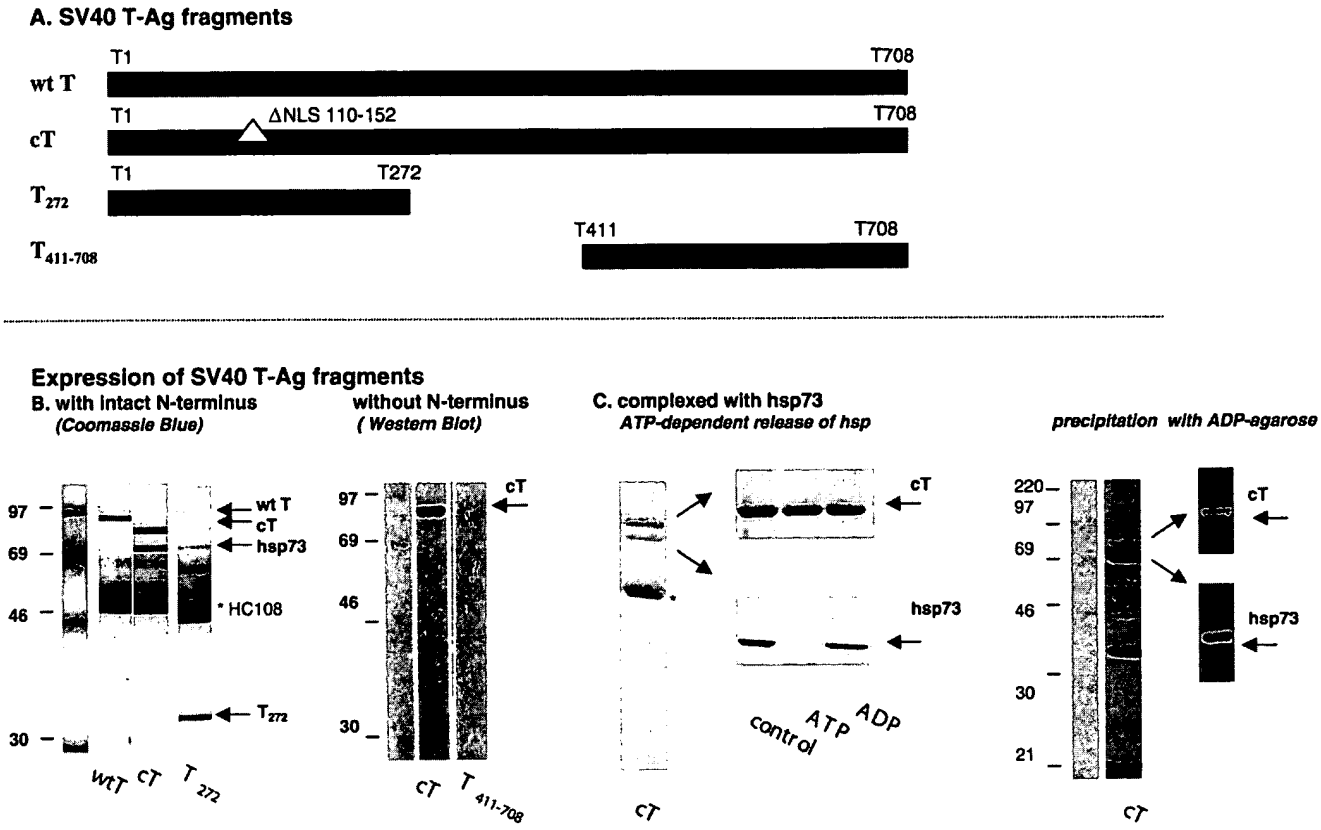


Figure 1. Expression of wild-type and mutant SV40 T-Ags. A) Map of SV40 wt T-Ag (wt T), cytosolic cT-Ag with a deletion of the nuclear localization signal (cT) and the N-terminal T_{272} and C-terminal $T_{411\text{-}708}$ fragments. B, C) The respective antigen-encoding sequences were cloned into the pCI and BMGneo expression vectors and tested in stable and transiently transfected cells. **B)** Stable transfected RBL5 cells were lysed and immunoprecipitated with the mAb Pab 108 followed by sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining of the gels (left panel). Stable transfected RBL5 cells were lysed in an SDS-containing buffer, and lysates were analyzed by SDS-PAGE followed by Western blotting, using a polyclonal rabbit anti-T-serum (right panel). **C)** Stable cT-Ag-expressing cells were lysed and immunoprecipitated with Pab 108 (lane cT showed the Coomassie Blue stained gel). Protein A sepharose-coupled antibody-cT-hsp complexes were either untreated or treated with 5 mM ATP or ADP (see refs 6 and 7) and washed, and antibody-bound material was analyzed by T-Ag- (upper panel) and hsp73-specific Western blots (lower panel). Cells were labeled with ^{35}S-methionine for 60 min, lysed, and precipitated with ADP-agarose. Purified precipitates were analyzed by SDS-PAGE and fluorograghy of the gels (lane cT). In addition, nonlabeled precipitates were analyzed by T-Ag- and hsp73-specific Western blots. The positions of hsp73 and the respective T-antigens are indicated.

Fig. 2

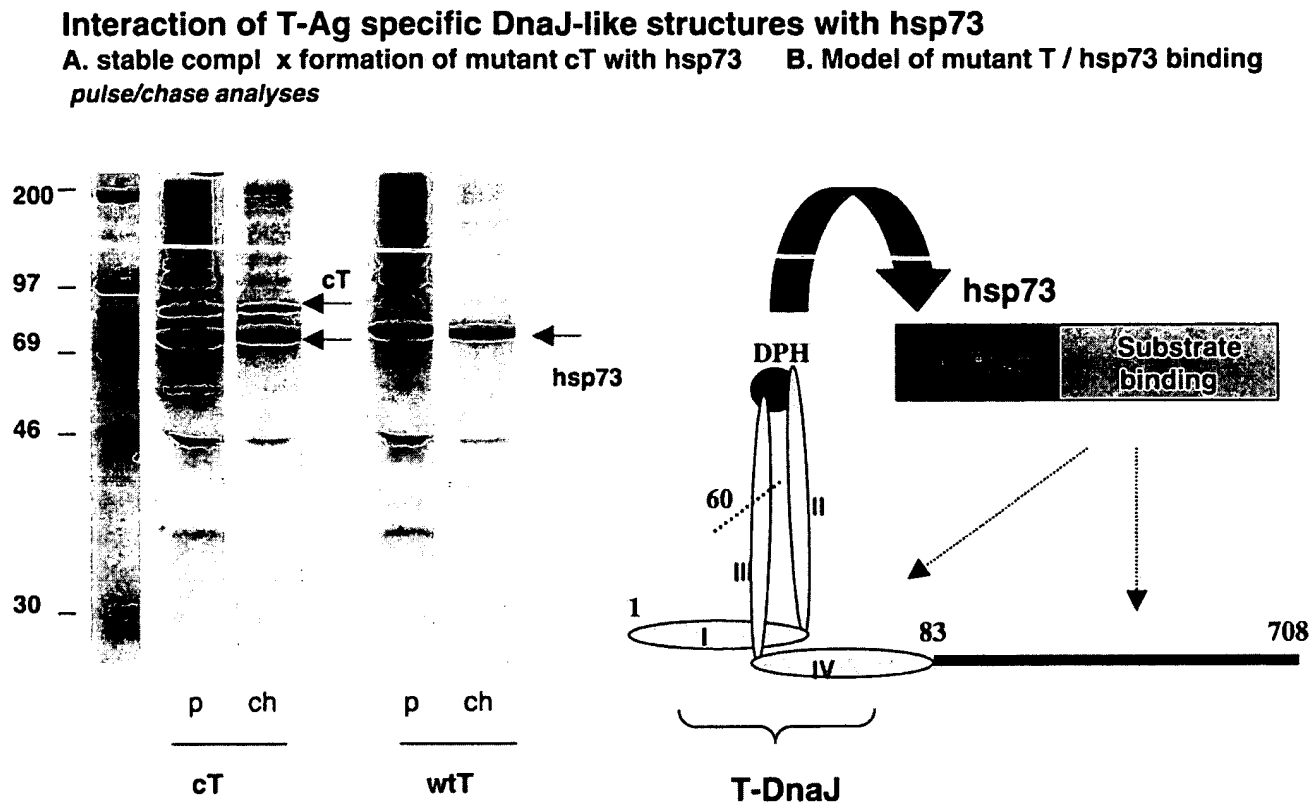


Figure 2. Stable association of cT-Ag, but not wt T-Ag and cellular proteins with hsp73. A) Stable transfected cells were labeled with ^{35}S-methionine for 15 min and chased for 60 min before extraction and immunoprecipitation with anti-hsp73 mAb SPA 815 (see refs 6 and 7). The positions of hsp73 and cT-Ag are indicated. **B)** Schematic representation of hsp73 binding DnaJ homologous region of SV40 T-Ag.

Fig. 3

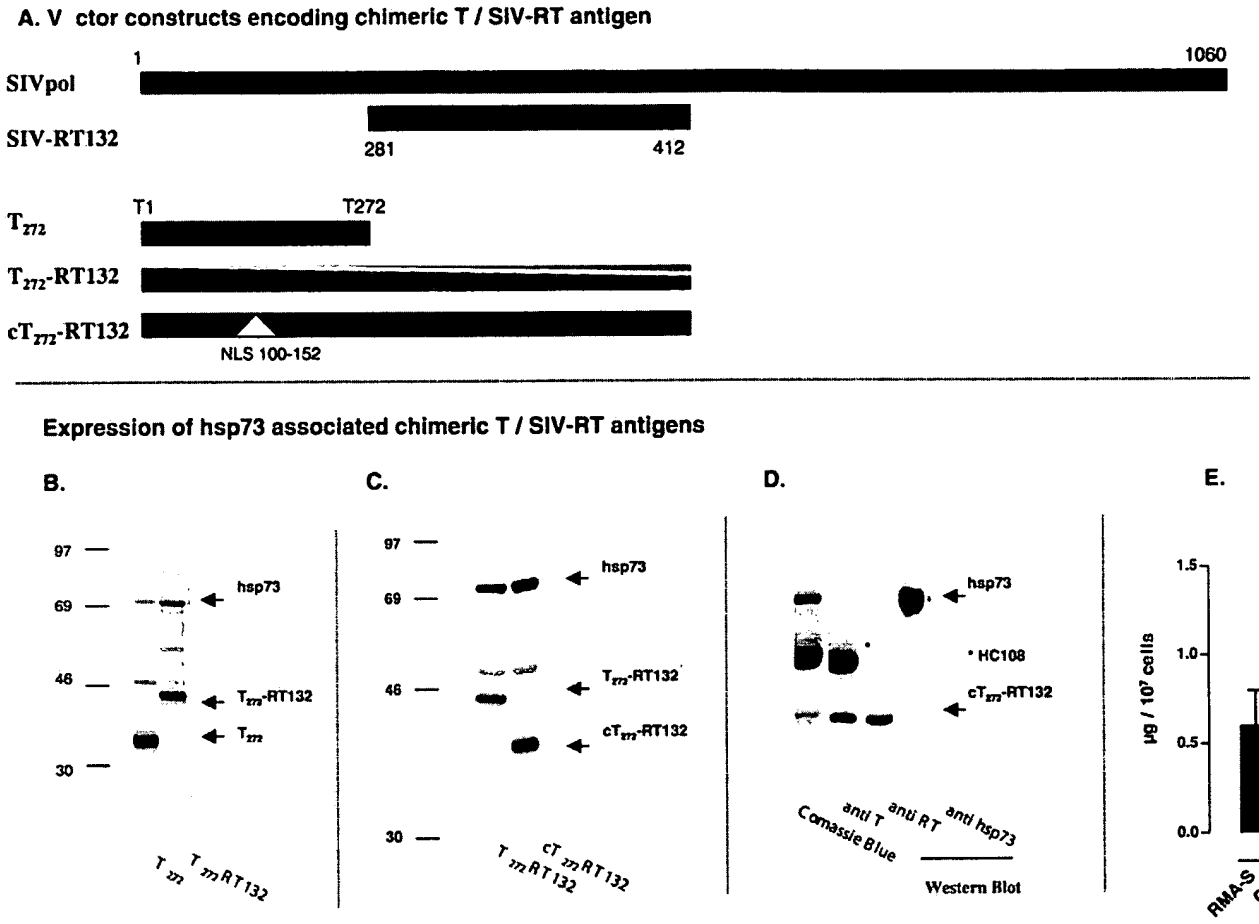
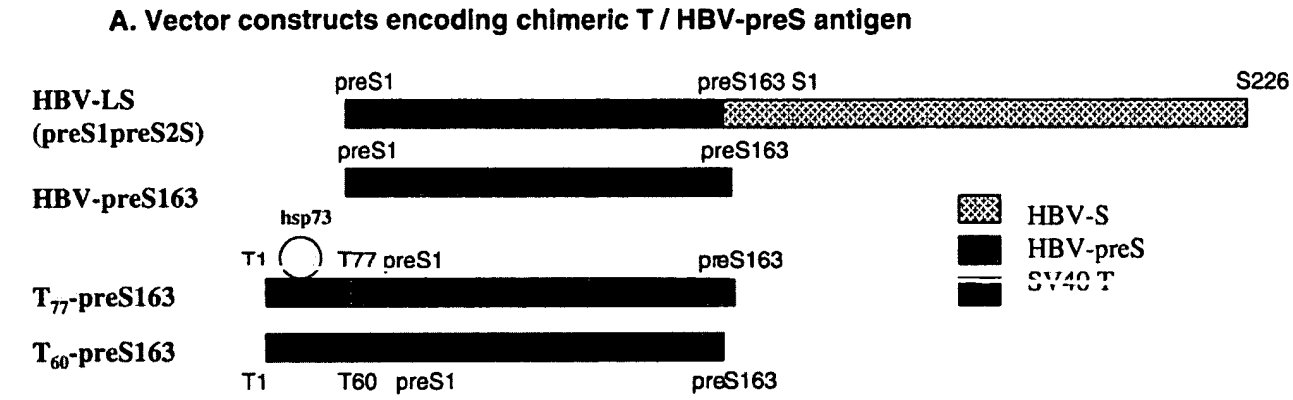


Figure 3. Expression of a 132aa residue SIVpol-derived reverse transcriptase (RT) unit using a novel hsp73-binding, SV40 T-Ag-derived expression system. A) Maps of SIVpol, the SIVRT132 fragment, and its fusion to T_{272} or cT_{272} fragments. **B, C)** LMH cells were transiently transfected with pCI/T_{272}, pCI/T_{272}-RT132, or pCI/cT_{272}-RT132; labeled with ^{35}S-methionine; extracted; and immunoprecipitated with anti-T-Ag mAb Pab 108 and processed for SDS-PAGE followed by fluorography of the gels. **D)** Stable T_{272}-RT132-expressing RBL5 cells were extracted, immunoprecipitated with anti-T-Ag mAb, and analyzed in Coomassie Blue stained gels or by T-, RT-, and hsp73-specific Western blots. **E)** Stable cT_{272}-RT132-expressing RMA-S, CHO, or Meth-A cells were extracted, immunoprecipitated with anti-T-Ag mAb Pab 108, and analyzed in Coomassie Blue stained gels. The amounts of fusion proteins were quantitatively determined as described in Materials and Methods. The positions of hsp73, and the respective T- and T-RT antigens are indicated.

Fig. 4



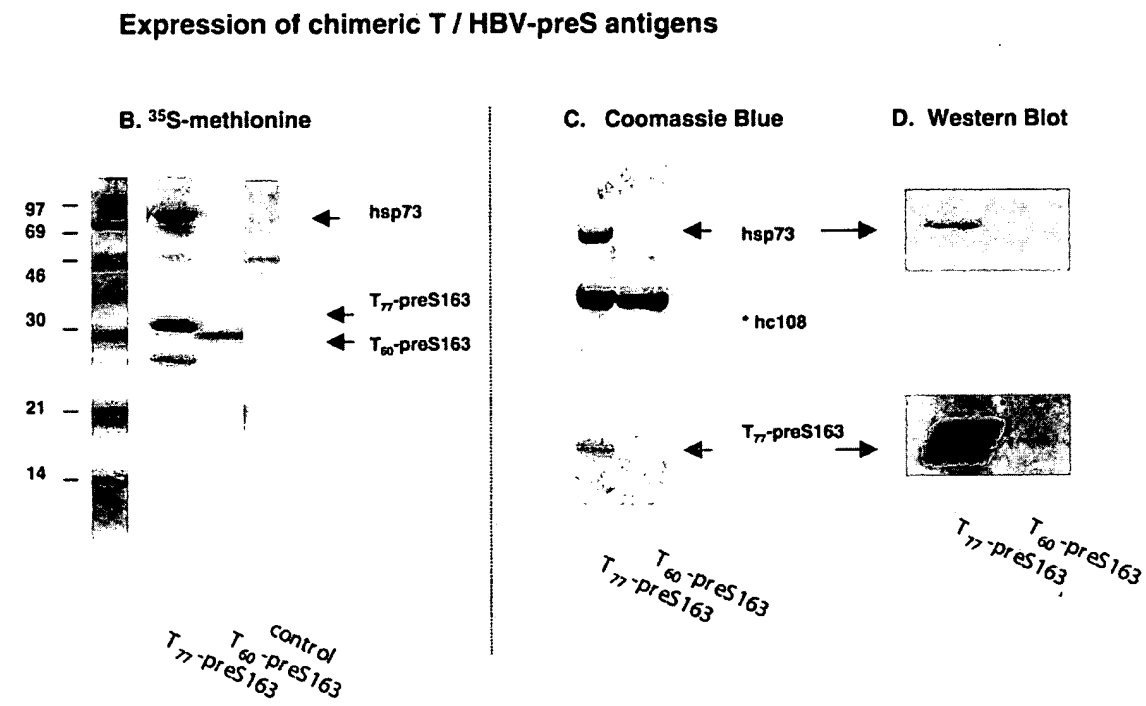


Figure 4. Characterization of the minimal hsp73 binding structure. A) Maps of hepatitis B virus large surface antigen (preS1-preS2- S), the preS domain, and its fusion constructs to T60 or T77. LMH cells were transiently transfected with pCI/T_{60}-preS163, pCI/T_{77}-preS163, or noncoding pCI vector; labeled with ^{35}S-methionine; extracted; and immunoprecipitated with anti-T-Ag mAb Pab 108 and processed for SDS-PAGE followed by fluorography of the gels **(B)** or by Coomassie Blue staining **(C)**. Nonlabeled immunoprecipitates of transfected cells were analyzed by hsp73-specific Western blotting, using the polyclonal anti-hsp73 rabbit serum SPA816 (Stress Gen) **(D)**.

Fig. 5

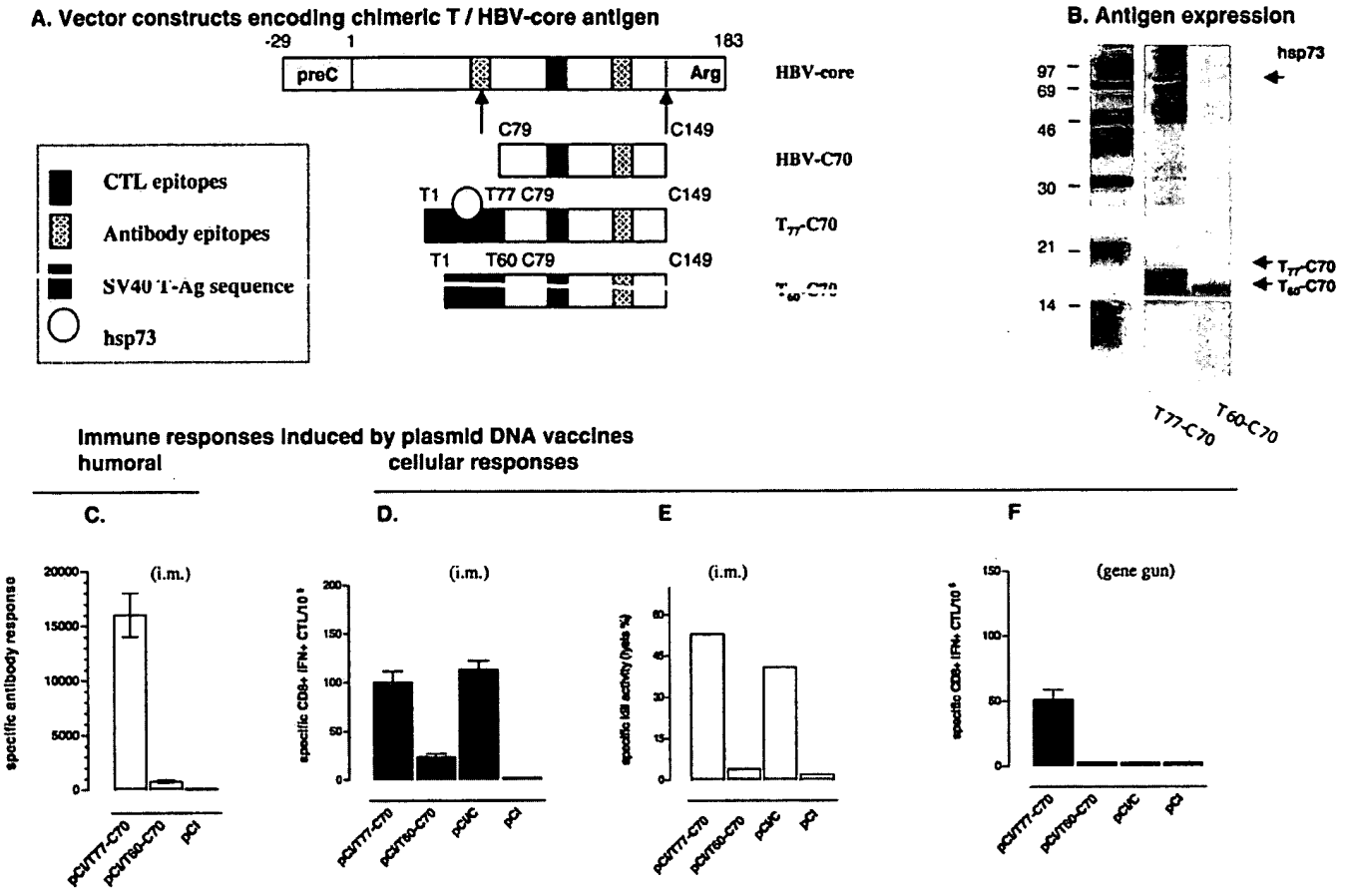
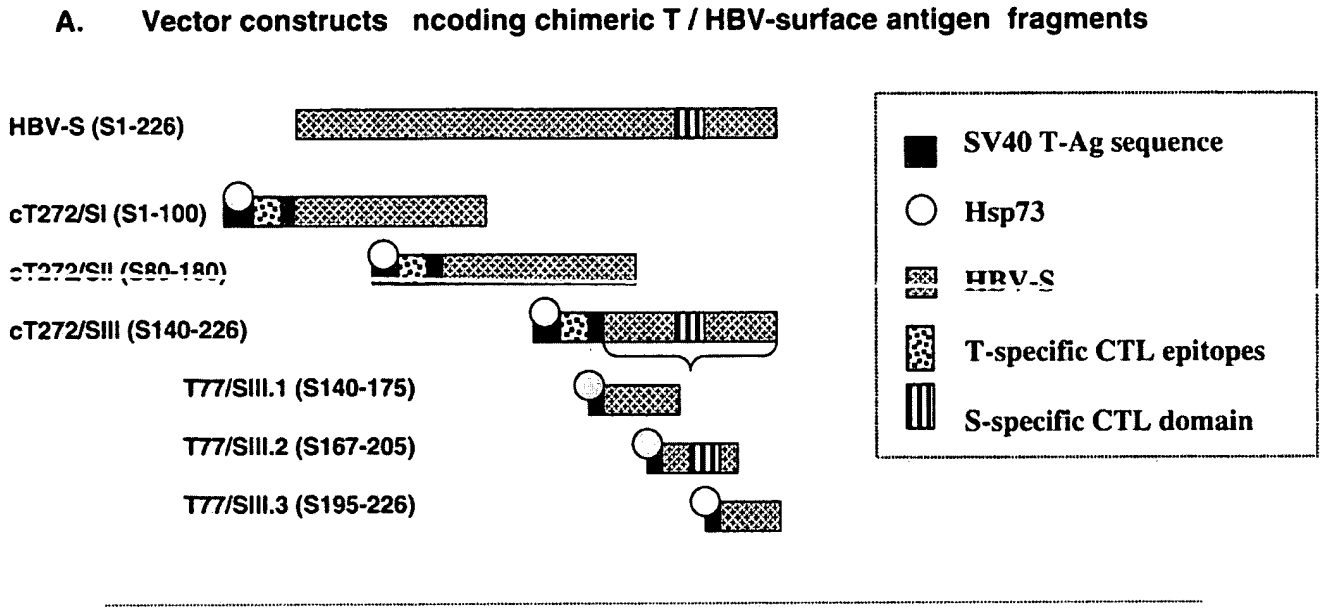


Figure 5. Priming HBcAg-specific cytotoxic T lymphocytes (CTL) and antibody responses. A) Maps of the hepatitis B virus core gen. The C79-149 domain and its fusion constructs to the T60 or the T77. **B)** LMH cells were transiently transfected with pCI/T_{60}-C70 or pCI/T_{77}-C70, labeled with ^{35}S-methionine, extracted, immunoprecipitated with anti-T-Ag mAb Pab 108, and processed for SDS-PAGE followed by fluorography of the gels. BALB/c mice were vaccinated into the tibialis anterior muscles (i.m.). **C–E)** With 100 μg plasmid DNA or intradermally (i.d.). **F)** (using the gen gun) with 2 μg plasmid DNA of the indicated vectors: pCI/T_{77}-C70, pCI/T_{60}-C70, pCI/C, or the control pCI DNA. Serum antibodies were obtained 5–8 wk postimmunization and analyzed for HBcAg-specific IgG serum antibodies in ELISA. The mean titers of antibodies in sera of threed to six mice per group are shown **(C)**. Spleen cells obtained 11 days postvaccination were restimulated for 5 h with HBcAg-expressing P815/C cells. T cells were surface-stained for CD8 and intracellularly stained for IFNγ. We determined the frequencies of $CD8^+$ $IFN\gamma^+$ CTL per 10^5 $CD8^+$ spleen cells by flow cytometry (FCM) analyses. Restimulation with nontransfected P815 cells was used to determine nonspecific "background" frequencies. The mean number of $IFN\gamma^+$ $CD8^+$ T cells/10^5 $CD8^+$ spleen cells ±SD of three to four individual mice are shown **(D, F)**. Alternatively, spleen cells obtained from immune mice 2–4 wk postvaccination were specifically restimulated *in vitro* for 5 days with HBcAg-expressing P815 transfectants and tested for specific cytotoxicity in a 4-h ^{51}Cr-release assay. Mean specific lysis values (in triplicate) at an effector/target (E/T) ratio of 20 are shown. The nonspecific lysis of control P815 (<5%) was subtracted **(E)**.

Fig. 6



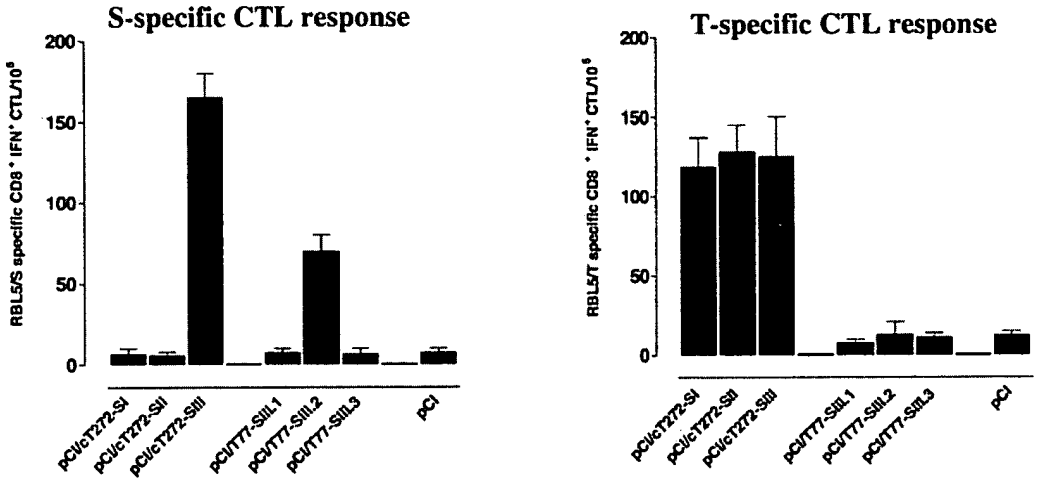


Figure 6. Priming HBsAg-specific cytotoxic T lymphocyte (CTL) responses. A) Construction of vectors expressing hsp-associated S-fragments. The first set of expression plasmids contain the N-terminal, hsp73-binding cT_{272} fragment fused in frame to the sequences $S_{1\text{-}100}$ (SI), $S_{80\text{-}180}$ (SII), and $S_{140\text{-}226}$ (SIII). The second set consists of the N-terminal, hsp73-binding unit (T_{77}) fused in frame to the sequences $S_{140\text{-}175}$ (SIII.1), $S_{167\text{-}205}$ (SIII.2), and the $S_{195\text{-}226}$ (SIII.3). **B)** Priming of specific CTL responses. C57BL/6 mice (H-2^b) were vaccinated i.m. with 100 μg/mouse plasmid DNA of the pCI/cT272-SI, pCI/cT272-SII, pCI/cT272-SIII, pCI/T77-SIII.1, pCI/T77-SIII.2, or pCI/T77-SIII.3 vectors, or control pCI DNA. Spleen cells obtained 14 days postvaccination were restimulated for 5 h with S-expressing RBL5/S cells (left panel) or with SV40 T-expressing RBL5/T cells (right panel). T cells were surface-stained for CD8 and intracellularly stained for IFNγ. We determined the frequencies of $CD8^+$ $IFN\gamma^+$ CTL per 10^5 $CD8^+$ spleen cells by FCM analyses. Restimulation with nontreated/nontransfected RBL5 cells was used to determine nonspecific CTL activation. The mean number of $IFN\gamma^+$ $CD8^+$ T cells/10^5 $CD8^+$ spleen cells ±SD of three individual mice is shown.

ATTACHMENT E

DNA vaccines expressing antigens with a stress protein-capturing domain display enhanced immunogenicity

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Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; H, histocompatibility; MHC, major histocompatibility complex; Hsp, heat schock protein; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; HPV, human papilloma virus; SV40, simian virus 40; T-Ag, large T (tumor) antigen of SV40; cT-Ag, cytoplasmic T-Ag (with a deleted nuclear localization sequence); wt, wild-type; gp, glycoprotein; IFN, interferon; TNF, tumour necrosis factor; NK, natural killer; ELI, expression library immunization; TAP, transporter associated with peptide translocation from the cytosol to the endoplasmic reticulum; ER, endoplasmic reticulum; GFP, green fuorescent protein; eGFP, enhanced GFP; OVA, ovalbumin;

Summary: An expression system for DNA vaccines is described that produces a product in which an N-terminal, viral J domain (that captures heat shock proteins, Hsp) is fused to antigen-encoding sequences (of various length and origin). This system supports strikingly enhanced expression and half-life of chimeric antigens (of >800 residues in length). These antigens are delivered with intrinsic adjuvant activity through the bound Hsp. We describe the design of vectors, and their expression of chimeric antigens. Using these vectors as DNA vaccines, we characterized the immunogenicity of the products of this expression system, especially for $CD8^+$ T cell precursors. We finally discuss areas of vaccine research to which the new technology may provide useful contributions.

Summary

1. Introduction

2. Stress protein-binding can facilitate expression of mutant or artificial proteins
   - The Hsp70-binding J domain of (DnaJ-related) Hsp40 proteins
   - Enhanced expression and half-life of mutant T-Ag with a J domain
   - Association of Hsp73 with J domain-expressing T-Ag variants
   - Unusually stable association of mutant T-Ag with hsp73
   - Hsp73 capture facilitates expression of large fusion antigen constructs with an N-terminal T-Ag J-domain

3. Role of Hsp molecules in vaccines
   - *Antigen*: microbial Hsp are immunodominant antigens
   - *Adjuvant*: Hsp as adjuvants in vaccines
   - *Chaperone*: Hsp as chaperones in vaccines that facilitate T cell priming

4. Incorporation of Hsp-capturing domains into DNA vaccines can increase the amount and immunogenicity of antigenic information they encode

Enhancing and modulating T cell immunity
- Associating hsp to endogenous antigen facilitates its TAP-independent processing for MHC class I-restricted epitope presentation
- Hsp73-bound chimeric antigen can be designed to prime mono- or multi-specific $CD8^+$ T cell responses
- Cross-presentation of epitopes by expressing hsp-associated antigen

Enhancing and modulating B cell immunity
- Endogenous, hsp73-bound antigen is visible to antibody-producing B cells
- Hsp/antigen complexes can focus an antibody response to weak, antibody-binding epitopes

5. Potential applications for DNA vaccines expressing hsp-capturing chimeric antigen

   Identification of antigenic, stimulating and suppressive domains or epitopes

   Expression library immunization (ELI)

   Construction of polyvalent, chimeric vaccines

   Generation of monospecific probes

   Facilitating $CD8^+$ T cell crosspriming by tumour cells

   Overriding low responder status

6. Concluding remarks

Introduction

The introduction of plasmid DNA-based vaccines into experimental research has greatly simplified the design and preclinical testing of alternative ways to enhance the immunogenicity of antigens. This is because it is much simpler to combine multiple DNA fragments (that represent different antigenic or immune-stimulating sequences) by recombinant DNA technology than to use heterologous expression systems and elaborate purification schemes to preparatively isolate protein-based vaccine candidates. To facilitate codelivery of an extended spectrum of antigenic and immune-stimulating information, complex expression systems using polycistronic cassettes (1, 2), bidirectional promoters (3, 4), long fusion constructs (5, 6) or multiple, independent transcription units on a single plasmid have been incorporated into DNA vaccines. Expression system design is thus the central challenge in the construction of DNA vaccines that deliver immunogens for eliciting a broad range of specific immune effector functions.

A protein (domain) expressed by a DNA vaccine *in situ* often displays the native conformation (with the relevant posttranslational modifications such as *e.g.* glycosylation, proteolytic processing, lipid conjugations) required to stimulate antibody responses to conformational epitopes, a feature essential for eliciting many neutralizing, anti-viral humoral immune responses. As it delivers antigenic information into the protein synthesis machinery of the cell (comparable to virus infection), genetic vaccination is also exceptionally potent in stimulating T cell responses. When combined, DNA vaccine formulations can thus efficiently co stimulate cellular and humoral immunity. The delivery of antigenic information to different compartments of the specific immune system by DNA vaccines can be further enhanced and modulated by codelivering immune-stimulating molecules or targeting the antigen to particular subcellular compartments, presenting cells or tissue compartments. For example, cytokine or costimulator molecules have been shown to enhance the immunogenicity of antigens encoded by DNA vaccines (7-19). The access of *in situ* expressed antigens to

immune recognition can be facilitated by expr ssing them as secreted antigens, by targeting them within the cell to relevant processing compartments, or by targeting them outside the cell to the relevant secondary lymphoid tissues and/or antigen-presenting cells (APC) (20-25).

We summarize in this review our data on a novel system to construct DNA vaccines in which antigenic domains are co expresses with a heat shock protein (Hsp)-capturing domain. Fusion of an N-terminal, viral J domain that captures hsp70 to antigen-encoding sequences (of various length and origin) supports stable expression of chimeric antigens with T cell- or B cell-defined epitopes of >800 residues in length, and delivers the chimeric antigens with novel immunogenic features. The two major advantages of the system are that it (i) enhances expression and half-life of the protein fused to the Hsp-binding domain, and (ii) provides intrinsic adjuvant activity. We briefly summarize the relevant features of the expression system, and put it into context to the biochemistry and immunobiology of Hsp molecules. We describe the construction of the vector, and the pattern of expression of the chimeric antigens that they encode before giving an update on the immunogenicity of antigens produced in this system by DNA vaccines (as characterized in different *in vitro* and *in vivo* readouts in mice). Finally, we indicate areas of DNA vaccination for which this technology may provide useful contributions.

Stress protein binding can facilitate expression of mutant or artificial proteins

The Hsp70-binding J domain of (DnaJ-related) Hsp40 proteins. Hsp40 proteins are a family of cochaperones that bind non-native conformations of polypeptides. The progenitor of the Hsp40 family, to which all its members still show striking homology, is the DnaJ molecule of *E.coli*. Hsp40 molecules colocalize with Hsp70 molecules in many subcellular compartments.

Hsp40 molecules and their viral homologues regulate the activity of Hsp70 molecules by binding to an (unknown) regulatory site of the molecule through their J-domain (containing a conserved HPD motif). This elicits an intramolecular signal within the multi-domain Hsp70 protein that activates its ATPase activity in an adjacent domain. Hsp70 with ADP bound shows increased affinity for polypeptides; exchange of the ADP by an ATP releases the polypeptide from the Hsp70 substrate-binding site. Hsp70/protein complexes are involved in the synthesis, folding, translocation (through membranes), signal transduction, proteolysis and renaturation of proteins. Hence, Hsp40 stabilizes Hsp70/protein complexes by binding to a regulatory site that controls ATPase activity.

Enhanced expression and half-life of mutant T-Ag with a J domain. In contrast to most other proteins, mutant forms of SV40 T-Ag (with an intact N-terminus) show stable expression in eukaryotic cells (26, 27). From the lysates of metabolically labelled, (transiently or stably) transfected cells of different species or tissue origin, the antigen was immunoprecipitated using monoclonal antibodies (*e.g.* mAb PAb108 described in (28) binding the extreme N-terminus of the T-Ag. Without exception, this demonstrated that the mutant or truncated T-Ag that contained an intact J domain bound hsp73 and accumulated in cells to levels of 0.2 – 1 µg per $5x10^6$ cells (26, 27). C-terminal T-Ag fragments without an intact J domain could not be expressed at all. Furthermore, pulse chase studies indicated that the half-life of the mutant or truncated T-Ag proteins was >12 h (26, 27). Hence, mutant T-Ag is unexpectedly stable *in vivo* if it contains a J domain.

Association of hsp73 with J domain-expressing T-Ag variants. The association of cytosolic Hsp70 with mutant or truncated T-Ag from SV40 or other polyomaviruses has been reported (29-31). We have confirmed and extended these observations by showing that different mutant or truncated SV40 T-Ag variants accumulate within cells of different histotypes in tight

association with hsp (Fig. 1) (26). For the strong, non-covalent binding of hsp to the N-terminus of the SV40 T-Ag molecule, its N-terminal 77-residue J domain sequence is required. This is the minimal but essential requirement to achieve hsp-binding and intracellular stability of mutant T-Ag variants (32).

The *in situ* association with hsp correlates with the stability and immunogenicity of mutant T-Ag. Mutant T-Ag and hsp70 molecules are co precipitated from cell lysates using monoclonal antibodies directed against either T-Ag, or hsp70. ATP that reduces substrate-binding affinity of hsp70 (by displacing ADP) inhibits hsp-binding to mutant T-Ag in a dose-dependent manner. The N-terminal 77-residue domain of T-Ag thus mediates docking to, regulating of, and substrate binding of cytosolic hsp70 molecules. Using monoclonal antibodies specific for individual members of the murine hsp70 family, we identified hsp73 constitutively expressed in the cytosol as the only hsp70 molecules bound to the J domain of T-Ag. Hsp-stabilized expression system thus involves a particular member of the hsp70 family, hsp73, and operates under steady state. Antigen or hsp/antigen complexes could not be immunoprecipitated from the supernatant conditioned by (transient or stable) transfectants. Thus, these antigens are either not secreted by the cells that produce them, or are rapidly cleared from the extra cellular space once released by cells. Possibly, low amounts of T-Ag/hsp73 are released and rapidly (re)internalized by the same or neighbouring cells. It is difficult to demonstrate this experimentally.

Unusual stable association of mutant T-Ag with hsp73. Constitutively expressed hsp73 bind unfolded but not native polypeptides, and prevent premature protein folding during protein biosynthesis (33). Hsp73 discriminates folded from unfolded proteins. It recognizes motifs (preferentially localized in β-sheets) present on the average every 36 residues in the linear sequence of all cellular proteins. These motifs are not accessible to hsp73 recognition in the native conformation of the proteins. This recognition is stimulated by DnaJ-homologous

chaperones. The highly conserved J domain mediates the association of DnaJ-like chaperones with hsp73. The J domain is composed of four α-helices with helix II and III forming a finger-like structure that exposes the conserved HPD motif on the tip of the loop (Fig. 2) (34). Dissociation of hsp73/substrate complexes is triggered by ADP release that initiates a new cycle of ATP binding to the N-terminal domain of hsp73. Immunoprecipitation analyses of pulse/chase labelled cells with anti-hsp73 antibodies showed that hsp73 preferentially binds to long but not short polypeptides (Fig. 1). Small proteins fold more rapidly into native conformations and/or exhibit a low affinity for hsp73 as their number of potential hsp-binding motifs is low. Consistent with the proposed role of hsp73 in preventing folding of nascent proteins, hsp73 associates transiently with cellular proteins early during their synthesis, as is evident from analyses of wt T-Ag expression (Fig. 1). In contrast, association of hsp73 with mutant T-Ag is stable (Fig. 1) suggesting that the dissociation of hsp73 from mutant/truncated T-Ag is very slow. Wild-type T-Ag and mutant T-Ag contain identical Dna-J-like sequences. Thus, the unusually tight association of hsp73 with mutant T-Ag proteins can only be explained by conformational differences that favour stable hsp73 binding to mutant but not to wt T-Ag.

Hsp73 capture facilitates expression of large fusion antigen constructs with an N-terminal T-Ag J-domain. Many truncated T-Ag variants with an intact N-terminus are stably expressed *in vivo*. Only its N-terminal 77-residues are required for hsp73 capture and stable expression. From these observations, we developed expression vectors for large, chimeric fusion proteins containing an hsp73-binding, T-Ag-derived N-terminus (Fig. 3). In these chimeric proteins, fragments of different antigens with $CD8^+$ T cell- and/or antibody-stimulating determinants were cloned in frame behind the N-terminal J domain to generate hsp-bound, chimeric fusion proteins of varying length (26, 32, 35-41). Partial or complete sequences of different viral, tumor or model antigens have been successfully expressed in this system

(Table I). The system allows expression of antigenic domains that are either very instable as native protein domains (such as *e.g.* the preS domains of the hepatitis B surface antigen HBsAg) (35), or toxic for the producing cells (*e.g.* th X-protein of HBV). The longest constructs expressed in this system up to now are the hepatitis B polymerase (832 residues) (R.S., unpublished), and the Her2/Neu oncoprotein (>1200 residues) (R.Kiessling, Stockholm, Sweden, unpublished). We have not yet encountered a size limit for antigen expressed in the system.

An observation, reproduced with many of these chimeric antigens is that the longer the chimeric protein, the more hsp73 molecules it captures. This 'cis effect' seems to reflect cooperative hsp73 binding: hsp73 already bound to the J domain seems to facilitate binding of additional hsp73 molecules to neighbouring hsp binding sites on the same protein. As hsp-binding sites are present at intervals of every 36 residues in all proteins, this phenomenon is efficient and ubiquitous. Limitation of chimeric antigen expression through hsp73 depletion seems unlikely because the cell adjusts its hsp73 synthesis rate to the level of free chaperone (available for substrate capture) but not to the level of substrate-bound hsp73. The expression system thus allows *in situ* production of large chimeric antigens with a long half-life that accumulate in the producing cells in substantial amounts but apparently remains largely cell-associated.

Role of Hsp molecules in vaccines

Stress protein molecules have been incorporated into experimental, DNA- or protein (peptide)-based vaccines as either antigens, or chaperones, or adjuvants (Table II). Although it is often difficult to distinguish these three features of Hsp molecules present in vaccines, the published data support three distinct roles involved in the immunogenic or

immunogenicity-modulating effects of Hsp.

Microbial Hsp are immunodominant antigens. Immunodominant T cell reactivities to many bacterial and protozoan pathogens of man and mouse are directed against epitopes of Hsp molecules. These include *e.g.* αβ and γδ T cell reactivities to Hsp of mycobacteria (42-48), chlamydia (49), yersinia (50), malaria plasmodium (51), or leishmania (52). As Hsp molecules are conserved, cross reactivities between microbial Hsp epitopes and their host cell homologues are not unexpected. This has been shown *e.g.* for MHC class II-restricted Hsp epitopes that stimulate $CD4^+$ T cells (53), MHC class Ia-restricted Hsp epitopes that stimulate $CD8^+$ T cells (54), or MHC class Ib (Qa-1)-restricted Hsp epitopes that stimulate NK cell and T cells (55). It has often been speculated that T cells 'naturally primed' to microbial Hsp determinants and repeatedly boosted by a the respective autologuous Hsp determinants during inflammatory responses thereby providing 'help'. This is difficult to distinguish from the direct adjuvants effects of Hsp on the innate immune system (described below). In our system, we could not elicit humoral or cellular autoimmune responses to autologuous hsp73 bound to 'foreign' antigens, even when >800 residues of immunogenic, viral antigen information was delivered by DNA vaccination. The system thus does not prime autoimmune responses to Hsp triggered by 'help' from non-covalently associated, strong immunogens.

Hsp as adjuvants in vaccines. Different approaches involving protein- or DNA-based vaccine constructs have indicated an immune response-potentiating role of Hsp. (i) In a peptide vaccine-based approach, viral antigen (p24 of HIV), model antigen (OVA) or polytope peptide have been (N- or C-terminally) covalently linked to mycobacterial Hsp70 and injected at low dose (without further adjuvants) (56-60). These vaccines efficiently prime potent $CD4^+$ T cell and antibody, as well as $CD8^+$ T cell responses when the antigen and the Hsp molecule were covalently linked. Not the intact Hsp molecule, but only a 200 residue domain of this protein

was required for its adjuvant effect, indicating that its chaperone function (ability to bind peptide) is not critical (58). (ii) In a DNA vaccine-based approach, the immunogenicity of DNA vaccines was enhanced by fusing an antigen sequence (*e.g.* a viral E7 antigen, or a model epitope) to mycobacterial Hsp70 or Hsp65 (6). This strikingly enhanced the potency of eliciting $CD4^+$ T cell-independent, tumor-rejecting $CD8^+$ T cell responses. Similarly, recombinant adenovirus or RNA replicons were used to deliver a fusion construct containing the HPV16 antigen E7 fused to mycobacterial Hsp70 (61, 62). These vaccines primed $CD4^+$ and $CD8^+$ T cell- as well as NK cell-dependent response that could suppress tumor growth. (iii) Synthetic peptides were produced that contained an N-terminal sequence representing a high-affinity ligand for the peptide-binding site of Hsp70, joined C-terminally to a $CD8^+$ T cell-stimulating epitope (63). Vaccinating mice with these hybrid peptides complexed to mouse Hsp70 effectively primed specific, $CD4^+$ T cell-independent $CD8^+$ T cell responses that could reject tumors expressing the relevant antigen. As Hsp70 itself, the Hsp70/hybrid peptide complexes activated dendritic cells (DC) for costimulation and cytokine release, providing an adjuvant effect that could bypass T cell help. (iv) A secreted gp97 molecule was expressed in tumor cells in which the endoplasmic reticulum (ER) retention signal was replaced by the Fc portion of IgG1. This Hsp captures peptides in the tumor cell before it is released, activates DC and NK cells, and thereby greatly facilitates priming of tumor-rejecting, MHC class I-restricted T cell responses (64, 65). These data from unrelated systems point to a potent adjuvant effect of Hsp molecules. They do not provide a hypothesis on the mechanism of action of this adjuvant effect which would facilitate the rational design of vaccines containing these 'physiological' immune-stimulants.

Hsp molecules bind to myeloid cell surface receptors thereby activating APC. Surface receptors for Hsp molecules on macrophages and DC seem to mediate their specific binding and endocytosis (66-69). Although the exact nature of these receptors remains controversial, some candidates have been proposed. These include CD14 (70), the toll-like receptors TLR2 and TLR4 (71), the α2-macroglobulin receptor CD91 (72, 73), and the scavenger receptor

LOX-1 (74). As the hsp70-binding entity is not unambiguously defined, the signal transduction pathway triggered by hsp binding is not mapped but a rapid rise in intracellular Ca^{++}, NFκB activation and MAP kinases ERK1/2 activation have been reported (70, 71).

Purified molecules of the Hsp60, Hsp70 and Hsp90 families from microbial or autologuous sources activate human and mouse macrophages, DC and endothelial cells (75-79), although a major contributing effect of contaminating microbial immune-stimulants has often been shown to be also involved (80). The potency with which Hsp from different families can activate APC differs considerably. Hsp molecules enhance uptake of antigen by DC (81), and upregulate expression of surface molecules required for adhesion (selectins, CD54), MHC-restricted presentation (MHC class I and class II molecules) and costimulation (CD86) (77, 79) of T cell responses. Hsp stimulation furthermore induces expression and release of IL-1β and IL-6 (75, 77), IFNγ, IL-12 and TNF (76, 79, 81), β-chemokines (82) and nitric oxide (83) by professional APC. In addition to DC maturation, Hsp molecules induce their migration (78). Hsp (not loaded with peptides) can also activate murine T cells *in vivo* and *in vitro* (84). These findings indicate the potential of Hsp molecules as activation signals for cells of the innate and specific immune system.

Hsp as chaperones in vaccines that facilitate T cell priming. Chaperones capture antigens, deliver them to processing, and load their resulting epitopes to MHC molecules. Hsp molecules bind substrate peptides with an extended conformation through hydrogen-bonding, hydrophobic contacts and/or salt bridges in the α-helical groove structure (33). Preferred substrates for hsp70 chaperones are hydrophobic residues that may include some basic (but no acidic) residues. Hsp73 seems to distinguish between the folded and the unfolded forms of the same protein, and binds only to the latter. Hsp70 molecules do not show the exquisite length and motif restrictions of peptide binding characteristic for MHC molecules. Hsp molecules have been implicated in pathways leading to MHC class I- as well

as class II-restricted presentation of epitopes from exogenous and endogenous antigens. Most research has focused on its effect on class I-restricted epitope processing and presentation.

Constitutively expressed Hsp molecules can enhance antigen processing/ presentation to $CD4^+$ T cells. Heat shock of B cells enhances processing of antigen (85) but decreases MHC class II-restricted presentation of their epitopes (86). Two different, constitutively expressed Hsp70 molecules have been shown to play a role in this processing pathway, *i.e.* PBP72/74 on the cell surface and in early endosomes (87-90), and Hsp73 in the cytosol and MHC class II-containing subcellular compartments (91). The mechanism of action of these chaperones in capturing, processing and/or exchanging peptides either against peptides of the invariant chain bound to MHC class II molecules in the MIIC compartment (92), or against peptides of recycling MHC class II molecules (93, 94) is unknown. A role of chaperones seems attractive in the currently favored model for processing and presentation of antigenic determinants by MHC class II molecules; this model proposes that binding of partially intact, unfolded antigen to MHC class II molecules precedes their subsequent trimming to fragments of a smaller size that proceeds until the final epitope size is reached with the antigen remaining bound to the MHC class II molecule (95).

Specific $CD8^+$ T cell immunity is elicited when Hsp molecules isolated from normal, virus-infected or cancer cells are injected into syngeneic or allogeneic mice. Similarly, Hsp/peptide complexes reconstituted *in vitro* (*i.e.* Hsp molecules purified from cells are freed of proteins/peptides they are associated with, and 'loaded' with antigenic peptides) efficiently prime $CD8^+$ T cells with specificity for minor H proteins (96), virus proteins (97-102), or tumor antigens (103-108). $CD8^+$ T cell responses primed in this way can reject tumors (106, 109), or protect from virus infection (100).

How Hsp molecules channel antigenic peptides to the MHC class I processing and presentation pathway is unresolved. Evidence has been presented for an 'exogenous' as

well as an 'endogenous' pathway. Peptides introduced into the cytosol of APC associated with Hsp are more efficiently presented to $CD8^+$ T cells than the same antigenic peptides introduced into the cytosol either in free form, or associated with albumin (110). Cytosolic Hsp70/90 complexes seem to carry different precursor peptides of an MHC class I-binding peptide than the ER-resident gp96 (107). gp96 and an unrelated ER-resident, peptide-binding Hsp molecule, calreticulin, capture peptides translocate into the ER lumen from the cytosol by the TAP (111, 112). Facilitating traffic of peptide-loaded MHC class I molecules from the ER to the cell surface in APC has also been suggested to involve Hsp (113). Hsp molecules may thus chaperone processing, transport of proteolytic fragments, loading to MHC class I molecules, and traffic of loaded MHC class I molecules to the surface.

Hsp may be even more efficient in priming $CD8^+$ T cell responses *in vivo* through an exogenous pathway. Hsp/peptide complexes injected as exogenous proteins without adjuvants efficiently prime $CD8^+$ T cell responses. Irrespective of the subcellular compartment, the Hsp are derived from, they seem to be all captured by similar receptors on the surface of APC (66, 67, 96, 112). Surface expression of gp96 on tumor cells facilitates cross-priming of specific, tumor-rejecting $CD8^+$ T cell responses (114). Under these conditions, either direct priming by DC capturing Hsp/peptide complexes, or cross-priming triggers specific T cell responses in these systems. Taken together, the data indicate that Hsp can facilitate the processing/ presentation pathway of exogenous or endogenous antigens.

Immunization with Hsp/peptide complexes induces rapid and prolonged specific activation of $CD8^+$ T cells (115). Priming $CD8^+$ T cell responses by Hsp-associated antigen is $CD4^+$ 'helper' T cell-independent (6, 58, 60). But $CD8^+$ T cell priming is dose-dependent, with high dose gp96/peptide vaccines leading to active and specific, $CD4^+$ T cell-dependent suppression of $CD8^+$ T cell immunity (109). Specific activation and clonal expansion of $CD8^+$ T cells in response to gp96/peptide vaccination depends on perforin-competent (cytolytic) NK cells and IFNγ; specific activation of this T cell response by this vaccine requires a perforin-

dependent positive feedback loop between NK cells and DC for sustained NK cell activation and clonal $CD8^+$ T cell expansion and differentiation (65). It is unclear if direct priming, or cross priming is the major pathway for T cell activation (67, 116). Th selectiv accumulation of Hsp73 in DC-derived exosomes, and their implication in the extracellular transfer of antigenic information to professional APC (117) may indicate a role in cross priming. Necrotic (but not apoptotic) cells may be particularly effective in transferring Hsp-associated immunogenic information to professional APC (118). Hsp-facilitated delivery of antigen thus elicits a multitude of responses of the innate (macrophages, DC, NK cells) as well as the specific ($CD4^+$ and $CD8^+$ T cell) immune system, the complexity of which remains to be resolved.

Incorporation of Hsp-capturing domains into DNA vaccines can increase the amount and immunogenicity of antigenic information they encode

Using a DNA vaccine approach, we tested in different antigen systems the immunogenicity of proteins produced in the hsp-facilitated expression system in mice *in vivo*. We analyzed the priming and boosting of humoral and cellular immune responses. Particular interest was focused on the specific stimulation of MHC class I-restricted $CD8^+$ T cell responses. The *in vivo* studies were complemented by *in vitro* studies to elucidate processing pathways that hsp/protein complexes access, and to follow cross priming (*i.e.* the transfer of hsp/protein complexes produced in tumor cells to DC). We summarize the relevant finding and put them in the context of the current knowledge on chaperone, adjuvant and specific antigen-stimulating activity of hsp. We begin by describing selected aspects of the $CD8^+$ T cell-stimulating effect of hsp/antigen complexes produced from a DNA vaccine.

Associating hsp to endogenous antigen facilitates its TAP-independent processing for MHC class I-restricted epitope presentation. One of the first observations we made in the system was that processing of endogenous, hsp-associated (mutant) T-Ag for D^b-restricted epitope presentation was TAP-independent (26). This was confirmed in studies using TAP-deficient cell lines and different constructs encoding truncated or mutant, hsp-capturing T-Ag variants (27). Although details on pathway and proteolytic degradation in TAP-independent processing of endogenous antigens are still under investigation, the published evidence on intracellular traffic of hsp73 suggests an alternative processing pathway that leads to the endolysosomal compartment. Many pathways deliver cellular proteins to late endosomes and/or early lysosomes for acid degradation by endopeptidases (cathepsin B, D, E, H, L, M, N, S, T) and exopeptidases (cathepsin A, B2, C, III; dipeptidyl aminopeptidase II; carboxypeptidase C). These pathways include endocytosis of (extracellular or membrane-bound) proteins, diversion of proteins traveling through the exocytic pathway, and non-selective processes (*e.g.* constitutive microautophagy or stimulated macroautophagy). In addition, hsp73-associated cytosolic proteins directly enter endolysosomes through a polypeptide import resembling chaperone-assisted protein transport into other organelles (reviewed in (119-121). This uptake is stimulated by ATP, involves the lysosomal membrane glycoprotein receptor LGP96 (122), is selective and saturable. Following import into endolysosomes, protein/hsp73 complexes are detectable within the lumen of the endolysosomes for hours. These data provide evidence that stable association with hsp73 allows endogenous antigen to access alternative routes for processing that generate MHC class I-binding peptides.

Hsp73-bound chimeric antigen can be designed to prime mono- or multispecific $CD8^+$ T cell responses. Used in DNA vaccination, the expression system described above is suitable either to focus $CD8^+$ T cell responses to an individual epitope, or to coprime multispecific $CD8^+$ T cell responses to different epitopes. The following five points are potentially relevant

in this context. (i) DNA vaccination in itself is a potent technique to elicit $CD8^+$ T cell responses. (ii) The hsp73-facilitated expression system allows in frame fusion of either small (single epitopes), or large (polyepitope constructs) coding sequences to the J domain that are equally well expressed. (iii) The system allows incorporation of (natural or modified) sequences flanking an epitope to ensure or facilitate its processing. (iv) Evidence (described above) indicates that the 'endogenous' antigen construct can access alternative processing pathways of the cell that are expected to harbour different proteolytic systems that can potentially give rise to alternative epitope repertoires (123). (v) The selective incorporation of only certain fragments of an antigen into the chimeric construct allows the elimination of suppressive (immunodominant or suppressor cell-stimulating) epitopes from the construct (40). We have reported the usefulness of the system for mapping $CD8^+$ T cell-stimulating epitopes (37), for designing polytope vaccines with enhanced immunogenicity for the stimulation of multispecific $CD8^+$ T cell responses (41), for eliminating suppressive, $CD8^+$ T cell-stimulating epitopes (40), and for the characterization of processing efficiency regulated by flanking sequences (123). In combination with its 'intrinsic adjuvant activity' and its impressive 'carrying capacity', the five points listed above can be readily exploited to develop optimized vaccines to stimulate mono- or multispecific T cell responses of defined restriction/epitope specificity.

Cross-presentation of epitopes is facilitated by expressing hsp-associated antigen. Priming $CD8^+$ T cell responses by intramuscular DNA vaccination involves cross priming (124-129). In the T-Ag system we showed that more specific $CD8^+$ T cells (with identical epitope/restriction specificity) are elicited by a DNA vaccine that encoded hsp73-binding T-Ag variants than by a DNA vaccine encoding native, non-hsp-binding T-Ag (130). This observation can be generalized. Hsp-bound antigen expression facilitated priming of anti-viral, IFNγ-producing $CD8^+$ T cell responses in many, unrelated antigen systems because the

measured specific $CD8^+$ T cell frequencies are 3- to 8-fold higher after intramuscular vaccination with vector DNA encoding hsp-facilitated as compared to hsp-independent antigen expression (R.S., unpublished). The murine tumor cells P815 and Meth-A were transfected to express either (not hsp73-binding) wt T-Ag, or different hsp73-binding T-Ag variants (*e.g.*, cT-Ag, or chimeric cT_{272}-eGFP protein). *In vitro*, remnants from apoptotic wtT-Ag- or cT-Ag-expressing tumor cells were taken up and processed by DC, and K^b/D^b-binding epitopes of T-Ag were cross presented to $CD8^+$ T cells in a TAP-independent way. DC pulsed with remnants of transfected, apoptotic tumor cells cross-presented the T-Ag epitopes more efficiently when they processed ATP-sensitive hsp73/cT-Ag complexes than when they processed hsp-non-associated (native) T-Ag (130). *In vivo*, the growth of transfected $H\text{-}2^d$ Meth-A/cT tumors in semi-syngeneic $F1^{b\times d}$ hosts primed 3- to 5-fold higher numbers of T-Ag-specific, D^b/K^b-restricted and IFNγ-producing $CD8^+$ T cells than growth of transfected Meth-A/T tumors (130). These data indicate that cross priming $CD8^+$ T cells by transfer of antigenic material from an antigen-expressing donor cell (*e.g.* a tumor cell) to a professional APC is facilitated when the antigen is complexed to hsp. In addition to 'helping' T cell priming, associating an antigen during its expression from a DNA vaccine with hsp can facilitate B cell priming leading to enhanced serum antibody responses.

Endogenous, hsp73-bound antigen is visible to antibody-producing B cells. Using DNA vaccination, we observed that wt T-Ag (not stably associated with hsp73) induced a potent, multispecific $CD8^+$ T cell response but no antibody response while mutant cT-Ag (stably associated with hsp73) induced a similar multispecific $CD8^+$ T cell response but also an antibody response (35). Similarly, antigen fragments with B cell-stimulating determinants fused C-terminally to the hsp73-capturing J domain of the T-Ag stimulated serum antibody responses when the fusion protein was expressed from a DNA vaccine (35, 37, 39). This was unexpected in view of the reproducible finding that no secreted product is detectable in

medium conditioned by transfectants expressing high levels of hsp73-bound antigen. But it was compatible with the observation (described above) that hsp73 association facilitates cross-priming of $CD8^+$ T cell responses which includes antigen transfer from a producing cell to a processing and/or presenting cell (130). Hsp73/antigen complexes may be regurgitated (possibly at low levels) from the endolysosomes of antigen-expressing cell, and may be rapidly cleared thereafter from the extracellular medium by one of the hsp-capturing surface receptors of neighbouring cell (described above). As adjuvant (Hsp) and antigen are codelivered to APC, even low amounts of antigenic material may prime a humoral, and cross prime a cellular immune response. If correct, this observation would stress the exceptional enhancement in immunogenicity that antigenic determinants acquire by associating with the 'physiological adjuvant' hsp73.

Hsp/antigen complexes can focus an antibody response to weak, antibody-binding epitopes. The finding that chimeric antigens become 'visible' for B cells when expressed in tight association with hsp73 has been exploited to focus humoral immunity to a selected antibody-binding epitope of interest. This approach is simple when a linear epitope is targeted (37) but difficult when a conformational epitope stimulates the B cell response (39). This strategy has been employed to selectively generate antibody responses against subdominant or cryptic epitopes that are usually weak or undetectable in the presence of an immunodominant response (35). Codelivered with additional $CD4^+$ T cell-stimulating 'helper' determinant to TCR transgenic mice that contain only $CD4^+$ T cells specific for the respective MHC class II-restricted 'helper' epitope, this vaccination protocol is a potent tool to generate antibody responses even against 'weak' or 'cryptic' determinants that conventional vaccination protocols would miss.

Although many pieces of the puzzle are still missing for understanding the cell biology of hsp-facilitated antigen delivery to the cellular and humoral immune system, the available

evidence from this and many other experimental systems indicates many advantages of this form of antigen delivery in DNA vaccination. The discussed features of the system are summarized in table III. It is likely that the use of the system is not restricted to DNA vaccination. It could be equally well used in RNA-based vaccines and in recombinant virus vaccines. Furthermore, current efforts in the group are focussed on techniques for the efficient isolation of intact hsp73/antigen complexes from eukaryotic cell lines to use them as a protein-based vaccine (R.S., unpublished). There are thus different options for the exploitation of the expression system in vaccine designs. Furthermore, the available data indicate that the system has a chance to contribute to a number of current challenges facing vaccinology. In the following, we list some points to illustrate the potential of the technology.

Potential applications for DNA vaccines expressing hsp-capturing, chimeric antigen.

Identification of antigenic, stimulating and suppressive domains or epitopes. Sequences of different length from a complex antigen containing candidate epitopes (with or without their flanking sequences required for natural processing) can be cloned into the vector and tested for antigenicity by DNA vaccination of mice. Simple strategies have been successfully employed by us to narrow down long sequences from viral antigens to: (i) identify $CD8^+$ T cell-stimulating epitopes; (ii) map their minimal processing requirements; and (iii) exclude interference from responses to immune-suppressive epitopes. Through successive rounds of eliminating sequences followed by testing T cell responses they can still elicit, the optimal epitope generated by natural processing can be identified. This offers a number of advantages over conventional peptide mapping strategies.

Expression library immunization (ELI). ELI has been successfully used to identify antibody- and T cell-defined epitopes of pathogens (131-133). In extending the theme on epitope mapping described above, the vector system could be used to express antigenic fragments from an expression library. There are two advantages, the system may offer: (i) it provides adjuvant activity (that hence does not have to be co expressed by the fragment tested, or codelivered by a separate, immune stimulating entity); and (ii) many truncated fragments from the library could be rescued and expressed in hsp-bound form if poly-linker sites allowing fusion of the DNA fragment in the three alternative reading frames are incorporated after the hsp-capturing domain of the vector.

Construction of polyvalent, chimeric vaccines. Similar to polytope vaccines, the vector system supports expression of antigenic entities from very different sources into a single, chimeric construct. It has the advantage of (i) an extensive 'carrying capacity' (allowing expression of >800 residues); and (ii) introducing potentially antigenic peptides into alternative processing pathways. This offers a unique chance to study *in vivo* at different levels (expression, processing, MHC loading, surface expression, immune response priming) positive and negative interferences between different T cell responses that always operate in the generation of multispecific immune responses.

Generation of monospecific probes. DNA vaccination offers an attractive way to generate monoclonal antibodies or T cell lines with well-defined epitope specificities (134-139). The vector system, we presented, allows the expression of well-defined, antibody- or MHC-binding peptides generated in its natural context. It allows fine-tuning of priming by focussing the response to optimized antigenic determinants. Using the system, we have generated monoclonal antibodies and $CD8^+$ T cell lines against different determinants that would have been much more difficult using a peptide-based approach

Facilitating CD8$^+$ T cell cross priming by tumour cells. We described in a tumour model (130) that CD8$^+$ T cells are more efficiently cross primed when the antigen is expressed (by tumour cells) in association with hsp. CD8$^+$ T cell reactivity targeted to tumour-associated antigens may be more efficiently elicited by either a vaccine or growing tumour cell by coexpressing it in hsp-bound form.

Overriding low responder status. DNA vaccines are more effective than most alternative vaccination approaches in revealing subdominant CD8$^+$ T cell reactivities (140). Responsiveness can be established by either increasing the immunogenicity of the epitope (123, 141), or by eliminating immunodominant T cell responses (40, 123). Combining both strategies can optimize the immunogenicity of the individual components of a polyvalent vaccine.

Concluding remarks

Our interest in a novel expression system evolved into studies on the optimal design of DNA vaccines that proofed valuable tools to elucidate optimal conditions to prime CD8$^+$ T cell responses. We assume that expression systems and T cell immunology are at the core of DNA vaccine research. Even if most practical applications of this research will eventually lead away from DNA vaccination (to recombinant viruses or protein-based vaccines), the studies using DNA vaccines will be the central tool to elucidate many principles at the preclinical level.

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Table I

Expression of chimeric antigens with hsp-capturing J domains

fragment cloned behind the T-Ag J domain			
source	antigen(s)	fragment	length
HBV	Polymerase	1-832	832aa
HBV	Polymerase	1-380	380
HBV	Polymerase	351-620	270
HBV	Polymerase	585-832	248
HBV	X-Ag	1-154	154
HBV	core	79-149	71
HBV	S	1-100	100
HBV	S	80-180	100
HBV	S	140-226	86
HBV	S	140-175	36
HBV	S	167-205	39
HBV	S	195-226	32
HBV	preS1preS2	1-163	163
HBV	SII/^SI	S80-180 +S20-50	131
HBV	S/core	S80-180 + core 1-183	284
HBV	S/core	S80-180 +core110-183	174
HBV	S/eGFP	S140-226 +eGFP	316
HDV	L-Ag	1-214	214
HDV	L-Ag	1-82	82
HDV	L-Ag	73-152	80
HDV	L-Ag	141-214	75
HDV	L-Ag-eGFP	L-Ag +eGFP	444
HCV	core	1-150	150
SIVmac239	RT	281-412	136
marker proteins	eGFP	230	230
tumor dendritic cell	Her2 IL23-p19	1-1255	1255
synthetic	polytope	1-106	106

Table II

Role of hsp in vaccines

group	Hsp functions as	vaccination based on
A	antigen	DNA protein/peptide
B	chaperone	protein/peptide
	loaded with peptide *in vivo*	
	loaded with peptide *in vitro*	
C	adjuvants	DNA protein

Table III

Biochemical and immunological features of hsp-capturing, chimeric antigens

feature		antigens expressed as hsp-capturing, chimeric proteins
biochemical	stability	stable expression with half-life >12 h large chimeric constructs of >800 residues can be expressed expressed under constitutive (not stress-induced) conditions mainly cell-associated (not secreted)
	hsp binding	binds selectively to cytosolic Hsp73 molecules Hsp binding is ATP-sensitive long chimeric constructs bind additional hsp73 molecules in cis
immunological		does not prime autoimmunity to autologuous Hsp73 molecule
	T cells	enhances mono- and multispecific $CD8^+$ T cell priming by chimeric antigen processing and binding of epitopes to class I can be optimized TAP-independent processing for class I-restricted peptide presentation is supported facilitates cross priming by DC allows rapid identification of naturally processed, class I-binding epitopes
	B cells	makes 'endogenous' antigen accessible to B cells can focus antibody reactivity selectively to certain epitopes can make 'weak' antibody-binding epitopes immunogenic for B cells

ATTACHMENT F

1 - cT-HBV/X
2 - cT-HBV/pol
3 - cT-HBV/pol1/3
4 - cT-HBV/pol2/3
5 - cT-HBV/pol3/3
6 - cT-HBV/core79-149
7 - cT-HBV/S1-100
8 - cT-HBV/S80-180
9 - cT-HBV/S140-226
10 - cT-HBV/preS1preS2
11- cT-HBV/S80-180+S20-50
12 - cT-SIV/RT
13 - cT-Her2
14 - cT-eGFP
15 - cT-HDV/L
16 - cT-HDV/L-eGFP
17 - cT-HDV/S
18 - cT-HDV/L1-82
19 - cT-HDV/L73-152
20 - cT-HDV/L141-214
21 - cT-HBV/S80-180+core
22 - cT-HBV/S80-180+core110-183
23 - cT-HBV/S140-226+eGFP

24 – T77-HBV/X
25 - T77-HBV/pol
26 - T77-HBV/pol1/3
27 - T77-HBV/pol2/3
28 - T77-HBV/pol3/3
29 - T77-HBV/core79-149
30 - T77-HBV/S1-100
31 - T77-HBV/S80-180
32 - T77-HBV/S140-226
33 - T77-HBV/preS1preS2
34 - T77-HBV/S80-180+S20-50
35 - T77-SIV/RT
36 - T77-Her2
37 - T77-eGFP
38 - T77-HDV/L
39 - T77-HDV/L-eGFP
40 - T77-HDV/S
41 - T77-HDV/L1-82
42 - T77-HDV/L73-152
43 - T77-HDV/L141-214
44 - T77-HBV/S80-180+core
45 - T77-HBV/S80-180+core110-183
46 - T77-HBV/S140-226+eGFP
47 – T77-IL23 p19